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ZOOLOGICAL SCIENCES

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THE PROVENTRICULUS OF *CICINDELA SEXGUTTATA* FABRICIUS (COLEOPTERA : CICINDELIDAE)[1]

BY W. W. JUDD[2]

Abstract

The proventriculus of *Cicindela sexguttata* Fabricius is described. It is similar in form to that of the proventriculi of Adephaga described by authors. It is oval in shape, and its inner lining is organized into eight longitudinal folds bearing a coating of fine hairs.

Materials and Methods

Specimens of *Cicindela sexguttata* Fabricius were collected on gravel roads about London, Ont. during September, 1938. They were identified with a key to the genus *Cicindela* in Blatchley (4).

The digestive system was removed from a specimen, under water, with the aid of a binocular microscope and was immediately placed in Bouin's fixative. After being fixed for about 10 hr. the tissue was cleared of fixative by repeated changes of 70% alcohol and was stored in 70% alcohol. For histological study the digestive tract was sectioned (10μ) with a rotary microtome and the sections were stained with haemotoxylin and eosin.

Description

The proventriculus of *Cicindela sexguttata* is oval and is about 2 mm. long (P, Fig. 4). Its anterior end is embedded in the posterior end of the voluminous crop (C, Figs. 1, 4); its posterior end is narrowed and enters the anterior end of the mid-gut (MG, Fig. 4). The mid-gut crosses the abdominal cavity at right angles to the length of the proventriculus and its outer surface bears numerous finger-like caeca.

The body of the proventriculus is roughly rectangular in transverse section (P, Fig. 1). Its innermost lining is a thin sclerotized intima bearing a coating of fine hairs (CH, Fig. 2). Beneath the intima is the epithelial layer (EP). The whole lining of the proventriculus is organized in the form of eight longitudinal folds. Four of these are narrow partitions (CP), which, in transverse section, appear as finger-like processes projecting into the lumen of the proventriculus. Alternating with these are four ridges (CR) each

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[The December issue of Section D (Can. J. Research, D, 25: 175-215. 1947) was issued January 20, 1948.]

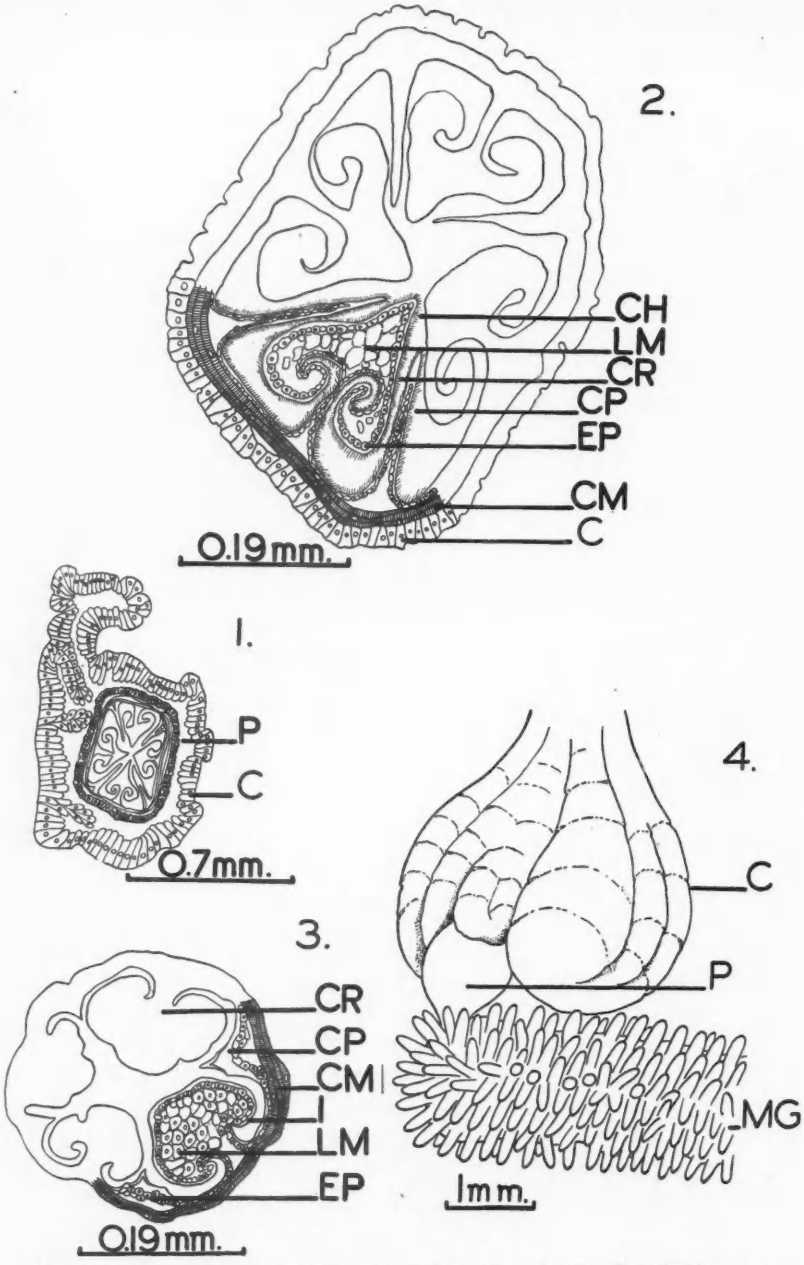

FIG. 1. *Transverse section of body of proventriculus and crop.* FIG. 2. *Transverse section of body of proventriculus.* FIG. 3. *Transverse section of posterior end of proventriculus.* FIG. 4. *Part of digestive tract showing crop, proventriculus, and mid-gut.*

C: crop; CH: coating of hairs; CM: circular muscle; CP: partition of proventriculus; CR: ridge of proventriculus; EP: epithelium; I: intima of proventriculus; LM: longitudinal muscle; MG: mid-gut; P: proventriculus.

attached to the wall of the proventriculus by a very narrow base. In transverse section these ridges are roughly triangular, one corner of the triangle projecting into the lumen, and the other two curled inward toward the base. The cavities of these ridges contain strands of longitudinal muscle (LM). The outer covering of the proventriculus is circular muscle (CM), three to four strands thick. Closely applied to the outer surface of the proventriculus is the epithelium of the crop.

The narrow posterior region of the proventriculus is roughly circular in transverse section (Fig. 3). The partitions (CP) are considerably wider in this region than in the body of the proventriculus, while the four ridges (CR) are rounded rather than triangular. The sclerotized intima (I) does not bear a coating of hairs.

Discussion

The foregoing description indicates that the structure of the proventriculus of *Cicindela sexguttata* is similar to that of the proventriculi of other beetles of the Adephaga described by authors. Descriptions of the proventriculus of four species of Carabidae were made by Bess (3), Judd (6), Schaefer (7), and Whittington (9). In these the proventriculus has eight longitudinal folds, as in *C. sexguttata*, but of these the four ridges are rounded rather than triangular in transverse section. In his description of the Dytiscidae Bordas (5) shows that this organ has eight teeth, four large and four small. Balfour-Browne (1, 2) also describes four "main" and four "intermediate" lobes in the proventriculus of the Dytiscidae. Thiel (8) concludes that in the Adephaga the proventriculus is a well developed structure with strong longitudinal and circular muscle.

Acknowledgment

The writer wishes to express his gratitude to Prof. J. D. Detwiler, University of Western Ontario, for his interest and guidance during the course of this work.

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CHITWOODIELLA OVOFILAMENTA GEN. ET SP. NOV., A NEMATODE PARASITE OF *GRYLLOTALPA*[1]

By M. A. Basir[2]

Abstract

Chitwoodiella ovofilamenta gen. et sp. nov. is described from *Gryllotalpa*. This new genus can be differentiated from other genera of the subfamily Thelastomatinae, by its long, striated buccal cavity, its characteristic filament-bearing eggs, and the position of the origin of the ovaries, both of which originate in the anterior region of the body from about the middle of the oesophagus.

In a previous paper (3) the writer has described five new nematode worms from mole crickets of the genus *Gryllotalpa*. During the continued study of the parasites of this group of insects, a new species, with an unusual method of egg production, was found. It belongs to the subfamily Thelastomatinae of the family Thelastomatidae (4, 5). It has a different structure from other representatives of this subfamily and cannot be accommodated in any known genus (1, 2, 6). Accordingly, the writer regards it as representing a new genus and a new species of the subfamily Thelastomatinae and the name *Chitwoodiella ovofilamenta* is proposed for it.

Description

Genus Chitwoodiella gen. nov.

Generic diagnosis: Thelastomatinae.

Male unknown.

Female with mouth opening circular, surrounded by a circumoral elevation and eight simple papillae. Amphids appear as small circular openings. Buccal cavity characteristic, very long and annulated. Oesophagus also very long, about one-fourth of the length of the body, consisting of a long corpus followed by a narrow isthmus and a posterior valvular bulb. Tail attenuated filiform. Vulva between middle and posterior third of body. Vagina short and directed anteriorly. Two ovaries, both anterior, originating in the oesophageal region a little behind the nerve ring. Uteri divergent. Eggs elliptical, organically connected with each other and enveloped by filamentous threads that arise in the form of a tuft from each pole; segmented before deposition and laid in the form of a chain.

Type species: Chitwoodiella ovofilamenta sp. nov.

Specific diagnosis: Chitwoodiella.

Male unknown.

Female 1.11 to 2.15 mm. long by 200μ in maximum width. Striations present only in the cervical region; striae 10 in number. First annule 10μ wide, remaining annules 5.3μ wide. Mouth circular, surrounded by a

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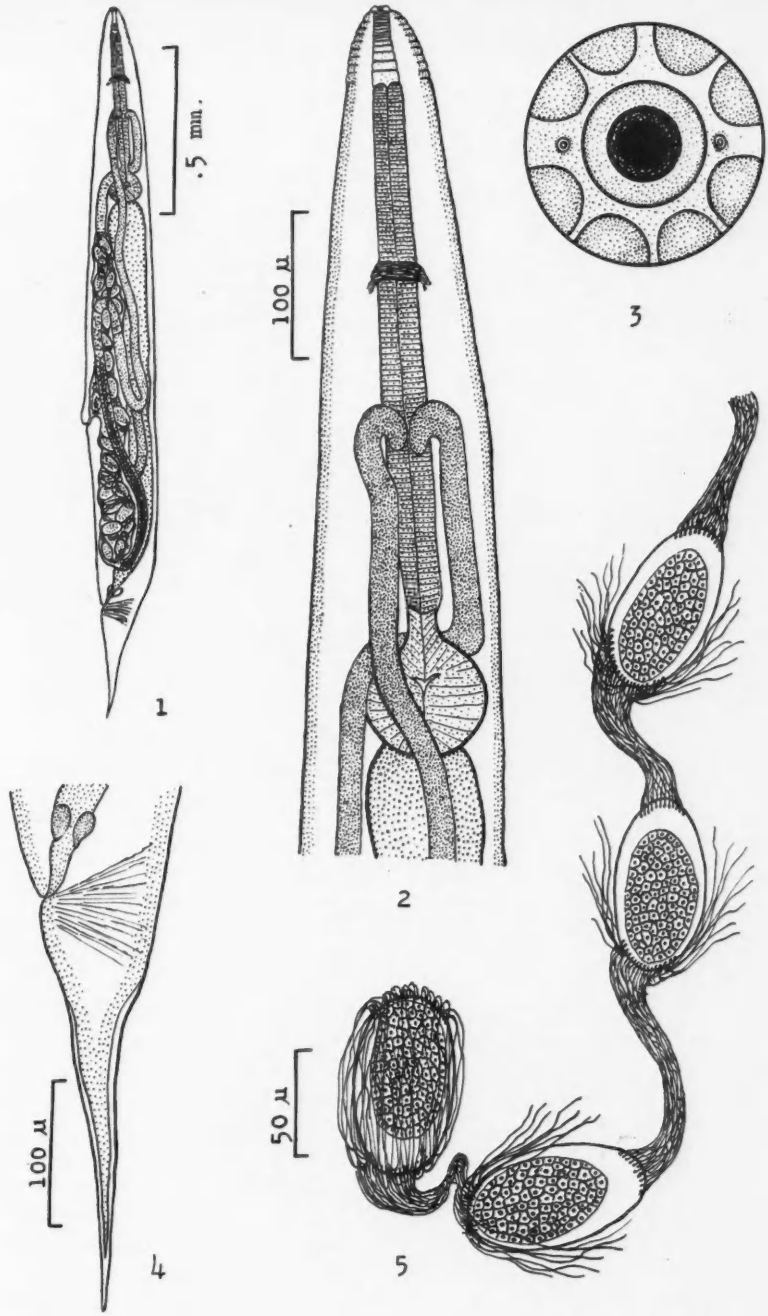

FIG. 1. *Female, entire, lateral view.*
FIG. 2. *Female, head, lateral view.*
FIG. 3. *Female, en face view.*
FIG. 4. *Female, tail, lateral view.*
FIG. 5. *A portion of the egg chain as laid by female (slightly pressed under the cover slip).*

circumoral elevation and eight simple papillae. Amphids present. Buccal cavity 53μ long, striated throughout its length, its anterior half being very finely striated and narrower in width than its posterior half; in the latter the striations are more distinct, wider apart, and fewer in number. Minimum width of the buccal cavity 10μ, maximum width, up to 18μ. Oesophagus 300 to 475μ long, consisting of a corpus 255 to 370μ long by 30μ at its maximum width, an isthmus 22 to 25μ long by 20μ wide and a posterior valvular bulb 60 to 80μ long by 60 to 85μ wide. Nerve ring 105 to 180μ from the anterior end of the body. Intestine enlarged anteriorly to form a cardia. Anus 150 to 290μ from the posterior end of body. Tail attenuated filiform. Vulva salient, projecting a little outwards, 1.25 mm. from the anterior end of body. Vagina short, directed anteriorly. Ovaries two, both anterior, originating in the oesophageal region a little behind the nerve ring. Uteri divergent. Eggs elliptical, 80μ long by 40μ wide, segmented before deposition, laid in the form of a chain being connected with each other and enveloped by threads that arise from each pole.

Host: *Gryllotalpa africana*.
Location: Intestine (rectum).
Type locality: Aligarh (North India).

Discussion

Egg filaments have been observed in a variety of nematodes belonging to different groups. They have been reported in species belonging to *Mermis*, *Tetrameres*, *Rhabdocona*, *Cystidicola*, *Metabronema* (7), *Binema* (10), and *Pseudonymous* (8, 9). However, in the family Thelastomatidae egg filaments have been recorded on two occasions only; one in *Pseudonymous* where a single filament arises and winds itself round the egg, and the other in *Binema* where filaments arise as polar tufts, and the filaments of two or more eggs form a loose network or shell, in which the eggs are lodged. They are laid in two or rarely in threes or even fours. In the present worm no such enclosure or secondary shell is formed; the eggs are laid singly, being connected with each other in the form of a chain. One peculiar thing about these eggs is that they are not separate but appear to be organically connected with each other by the filaments. One end of each of these filaments arises from one egg, and the other end is connected to the next egg. At the proximal pole of the egg, which passes out of the vulva last, the implantation of the filaments is light and only threads of the connecting tuft are attached. On the other pole, the implantation is thick and heavy, because, in addition to the attachment of the filaments of the tuft itself, numerous other filaments arise from this pole, curving round and enveloping the egg on all sides. These remain attached to the egg surface by some adhesive substance. This becomes clear on pressing the eggs, when the threads separate from the sides as shown in the figure. The connecting filaments are developed somewhere in the oviduct.

This raises a question that cannot be answered without further study, namely the method by which the eggs are laid. It seems probable that once egg laying starts, it cannot finish until the whole of one uterus is emptied

completely. Moreover, it seems probable that all the eggs from one uterus must pass out before the eggs from the other uterus can start to pass out. If this is not the case, the only alternative seems to be the breaking of the chain. However, the tuft of filaments is so heavy and stout that this seems to be improbable. When the eggs were pressed too heavily on a slide under a cover slip, the egg shells broke down but the connecting threads were still unbroken. This makes the possibility of their breaking up during normal egg laying even more improbable.

Apart from the slight resemblance in the presence of polar filaments on the eggs, this genus has little in common with the genera *Binema* and *Pseudonymous*. However, it shows some resemblance to the genus *Cephalobellus* Cobb, 1920, in the form of its body, but differs from it in the following points. In *Cephalobellus* the buccal cavity is very short, while in *Chitwoodiella* it is long and striated. In the latter, both ovaries arise in the oesophageal region, and the eggs bear polar filaments; in the former, one of the ovaries arises anteriorly and the other posteriorly, and the eggs have no polar filaments. The proportionate lengths of the oesophagus and tail are different in these two genera; in the former, both the oesophagus and the tail are comparatively much shorter than in the latter.

Because of the differences in the form of its long and striated buccal cavity, its characteristic filament-bearing eggs, and the position of the origin of the ovaries, this genus can be differentiated from all other genera of the subfamily Thelastomatinae. The writer believes that these differences are sufficient to justify the erection of a new genus.

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STUDIES ON THE HOUSEFLY (*MUSCA DOMESTICA* L.)

I. THE BIOLOGY AND LARGE SCALE PRODUCTION OF LABORATORY POPULATIONS[1]

By A. Wilkes,[2] G. E. Bucher,[2] J. W. MacB. Cameron,[3] and A. S. West, Jr.[4]

Abstract

Using the Peet-Grady method for propagating houseflies required as biological test animals, variations were observed that had considerable bearing on production and subsequent adult life. Differences in length of life, fecundity, time of emergence of the adults from puparia, and the onset of egg laying occurred between different populations of flies. Investigations were carried out to determine the extent and causes of the variations and to develop more suitable techniques for producing large numbers on a more accurately predictable basis. Variability in production of puparia was found to be due largely to the age of female stock and rate of fermentation in the rearing medium through their effects on egg hatchability and larval survival and excessive crowding caused by high temperatures. By using eggs from genetically-selected stock of known age and rearing in a temperature-controlled medium, production of flies was increased and maintained at a constantly uniform rate. A description is given of the equipment and methods used.

Introduction

The housefly, *Musca domestica* L., is often used as a biological means of determining the effectiveness of insecticides in the establishment of control measures for many insect pests. In 1932 the Peet-Grady Method was adopted as an official test, having been designed to provide a standard means of evaluating insecticidal sprays in association with an Official Test Insecticide as a basis for comparison. In 1938 the Large Group Modification of the Peet-Grady Method was officially adopted in which larger groups of flies are used as test units. Both methods are being used extensively at the present time. An outline of the methods is given in Blue Book, Soap and Sanitary Chemicals, 1943 (1).

As biological tests the Peet-Grady methods are subject to the variations normally found among any group of living animals. It is necessary, therefore, that all tests should be carried out under comparably standard conditions in order that reasonable agreement can be obtained in the evaluation of the results. To do this, definite practices are followed involving the use of representative units of flies reared and tested in a prescribed manner and under certain uniformly standard conditions. In the production and handling of the flies certain routine methods of procedure are always followed particularly with regard to the rearing medium, incubation of the larvae, separation

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of the pupae, and handling of the adults. Even under these conditions, however, the results are not always as uniform as might be desired. This was found to be particularly so in the present work.

During the current series of investigations large quantities of flies were required for test purposes. Throughout the studies, it was essential that a fixed and often large number of them should be available for testing on a predetermined date and that biological variations between the different populations were as small as possible. Using the Peet-Grady rearing methods a number of difficulties were encountered and consequently, studies were undertaken to determine the causes and the possibility of developing more suitable techniques for producing the flies. The purpose of the present paper is to present the results obtained in this phase of the work with the hope that they may be of value to others engaged in similar biological assays.

The General Method of Rearing

The method first used for the production of flies followed closely the standard Peet-Grady procedure. A stock of flies was obtained by placing 500 puparia of the 'Powell' strain in a standard fly rearing cage 12 in. × 12 in. × 12 in. in a well-illuminated room maintained constantly at 27° C. and 50% relative humidity. After emergence the adults were provided daily with a mixture of equal parts of water and milk in small stender dishes, two to each cage, in which were placed pieces of paper towelling. Eggs were deposited by the females either on the towelling or on the moist edges of the dishes. The eggs were removed each morning and agitated in water before being separated into lots for breeding by volumetric estimations from a calibrated pipette.

The medium used for rearing the larvae is a modification of Richardson's formula (Richardson (2)) and consisted of the following ingredients:

Soft wheat bran —four battery jars (approximately 15,000 cc.)
Alfalfa meal —two battery jars (approximately 7500 cc.)
Malt extract —50 cc.
Bakers' yeast cake—94 gm.
Water —three and two-thirds battery jars (approximately 13,600 cc.)

This mixture was placed in cylindrical battery jars measuring 6 in. in diameter by 9 in. high to a depth of within 2 in. of the top.

In each battery jar 2500 eggs were stirred lightly beneath the surface of the medium and the jar covered with a damp cloth. After three days the cloth was allowed to remain dry. On the morning of the ninth day of incubation, pupation of the larvae was almost complete, the puparia usually being formed in the dry upper layer of the medium. The top inch of medium was then removed, spread on trays and placed in front of a fan until it was sufficiently dry to effect a clean separation of the pupae from the medium by

inclining the tray in the direct path of the air stream. Usually the puparia from the different jars were mixed and the number determined by weight. Incubation of the puparia was carried out at 26.7° C.

Variations in Puparial Production

During the propagation of flies using the method indicated, considerable variation in production was encountered. The yield of puparia varied not only from one battery jar to another but also between different generations. This was equally true of the relative sizes of the puparia.

At a density of feeding larvae represented by the number hatching from 2500 eggs per battery jar the mean weight of the puparia was 19.5 gm. per thousand, the standard deviation being 1.5 gm. For every increase of 500 eggs per jar the weight per one thousand puparia decreased by approximately 0.8 gm. Thus, size was an index of crowding of the larvae during rearing and could be changed by unfavorable conditions in the medium. In many cases abnormally small puparia were produced when the temperature of the rearing medium became too high. In these jars the larvae crowded into the layer of medium in which the temperature was lower i.e. nearer their preferred temperatures, and were to be found in masses, usually around the outer walls of the jars.

On the basis of the number of puparia reared from battery jars 'seeded' with 2500 eggs comparisons could readily be made. Although, normally, after eight days of incubation pupation of the larvae in the jars was complete, approximately 2% of the larvae usually failed to pupate within the eight day period. By discarding the culture after eight days of incubation the loss of these larvae was not particularly noticeable, since adult emergence from the puparia they formed was abnormally low. Usually the presence of these late maturing larvae indicated unsatisfactory rearing conditions such as excessive crowding, lack of sufficient food, or low temperatures and they constituted less than 0.1% of the puparia reared.

In determining the number of puparia reared per jar, estimates were made on the basis of the total weight of puparia and the weight of at least two counted samples of 500. The error of estimate was found by experiments to be 0.5%. For purposes of comparison production was expressed as the number of puparia per battery jar unit of rearing medium either as whole numbers to the nearest 10 or as the mean weight per jar-unit of puparia.

The most striking variable encountered during the production of flies was in the number of puparia per unit-jar of rearing medium. This is illustrated in records taken from one generation of laboratory-reared stock.

Lot No. 3, mean total production per jar = 39.3 gm.
No. 7, mean total production per jar = 21.2 gm.
No. 7, Jar *a*, total production per jar = 29.4 gm.
No. 7, Jar *b*, total production per jar = 15.7 gm.
No. 7, Jar *c*, total production per jar = 18.5 gm.

The average weight per puparium was 0.0195 gm. This represents a maximum difference in the production of over 60%. From a study of the differences a number of factors were found to be involved, an outline of which is given under the headings indicated.

The Age of Parental Females

It was clearly evident that as the age of the ovipositing females increased the number of puparia reared per jar decreased. This is shown in Table I. In the table is listed the production of puparia reared from females from the 1st to 23rd day of oviposition in Generations 5 to 10. The age of the females may be calculated by adding three days to the day of laying in each case.

TABLE I

PRODUCTION OF PUPARIA FROM EGGS DEPOSITED BY FEMALES THROUGHOUT THEIR EGG-LAYING PERIOD

Days of laying	Number of samples (jars)	Mean production of puparia per jar (gm.)
1	5	38.3
2	19	39.2
3	18	34.6
4	17	36.3
5	13	35.2
6	15	29.6
7	20	30.3
8	21	32.3
9	17	25.5
10	19	29.8
11	15	27.6
12	14	28.0
13	13	21.8
14	10	20.5
15	12	21.0
16	9	17.9
17	7	16.4
18	4	10.1
19	2	13.0
20	5	18.3
23	2	18.3

A similar drop in production with increased age of the females was also demonstrated in a strain referred to as 'wild', of which the original stock was secured from a barn near Belleville. Lower yields of puparia were due chiefly to decreased hatching of the eggs; as the females became older increased

proportions of the eggs failed to develop. In Table II the mean proportion of hatched eggs calculated from 25 populations is shown for both strains. The drop in percentage of hatched eggs was greater in the Powell strain than in the 'wild' strain.

TABLE II

DIFFERENCES IN THE HATCHING OF EGGS AND THE AGE OF PARENTAL FEMALES

Day of laying	Percentage of eggs hatched		Day of laying	Percentage of eggs hatched	
	Powell strain	Wild strain		Powell strain	Wild strain
1	91.2	94.8	18	68.6	80.4
2	86.3	92.3	19	64.6	84.6
3	85.9	92.0	20	63.8	77.5
4	82.4	91.6	21	50.6	79.0
5	86.4	88.6	22	54.2	77.3
6	81.5	89.5	23	47.5	80.6
7	82.4	89.4	24	60.4	71.5
8	78.3	90.3	25	58.4	75.7
9	85.8	88.6	26	38.7	63.8
10	79.1	87.7	27	43.0	73.0
11	76.4	86.9	28	32.9	48.0
12	80.2	87.5	29	25.7	—
13	73.0	88.1	30	26.2	—
14	78.4	85.1	31	44.2	—
15	68.7	86.7	32	—	—
16	71.9	88.7	33	—	—
17	66.4	85.5	34	32.0	—

Since males have a shorter life than females, only a few were present in the cages on the 10th day of laying. To determine whether the reduction in hatched eggs was due to the absence of males and a consequent deposition of sterile eggs, males were removed from several cages after mating had occurred once, while in other cages fresh males were introduced after 10 days of laying. The results showed that the decrease of percentage of hatch was not caused by the absence of male flies. This conclusion was supported by observations made during egg sampling. Unfertilized eggs could be recognized on inspection by their translucent appearance. Such eggs were not more numerous towards the end of the oviposition period. It seems probable that a change occurred in the nutrition of the eggs associated with increasing age of the females, although changes in the diet of females produced no significant differences in the rate of reduction of egg hatch with age of the parental females.

Lower yields of puparia were due also to a shortened life of some larvae as the age of the parental females increased. The survival of larvae varied inversely with the days of laying. In Table III a summary of production is given for two populations of adult breeding stock. Eggs from these females were placed in rearing on each day of oviposition, at densities of 2500, 3000, and 3500 eggs per battery jar. The jars were prepared in triplicate.

TABLE III

PRODUCTION RECORDS FOR THE COMPLETE OVIPOSITION PERIOD OF TWO ADULT POPULATIONS OF POWELL FLIES

Period of egg deposition	Percentage of eggs hatched	Production of puparia per jar			Survival of larvae, %		
		Eggs per jar					
		2500	3000	3500	2500	3000	3500
Generation P 16							
Mean 1 to 10 days	79.6	1670	1890	2100	83.6	79.1	75.1
Mean 11 to 20 days	72.4	1120	1310	1230	61.9	60.1	46.4
Mean 1 to 20 days	76.2	1430	1620	1720	73.8	70.2	62.6
Generation P 14							
Mean 1 to 10 days	85.5	1630	2070	2200	76.4	80.9	73.6
Mean 11 to 20 days	72.5	1040	1240	1420	57.5	57.5	56.7
Mean 1 to 20 days	79.0	1330	1660	1810	67.0	69.2	65.1

From these results it is concluded that variations in production of puparia between different populations of flies is often a result of differences in the age of the females but the rate of drop of production was not rapid until after the 10th day of oviposition. By this time between 60 and 70% of the total number of eggs had been laid. Thus by destroying the breeding stock after 10 days of egg laying variation in production was reduced but not completely eliminated.

Survival of Eggs

Samples of eggs to determine the percentage hatch were taken on all lots before rearing and thus provided a means of estimating expected production. Two or more lots of 100 eggs were counted and placed on damp, white blotting paper, soft paper towelling, or black photographic paper in a covered watch glass and incubated at 26.7° C. After 24 hr., the unhatched eggs were counted and subtracted from 100. Large series of tests showed that the standard deviation of individual samples was $\pm$ 4% and that there were no significant differences in hatch between the eggs taken for sampling and those hatching in the rearing medium. During the first 10 day period of egg-laying all viable eggs hatched within 24 hr. but after 10 days about 7% of the eggs did not hatch until after 24 hr. of incubation.

Within any one population of flies the decrease in hatching of the eggs with increased age of the females as shown in Table II was not regular from day to day. The curve is characterized by having fluctuations above and below the smoothed line, the magnitude of which increased with the age of the females. In the Powell strain the fluctuations occurred at daily intervals, a day of high egg hatch followed by one of low hatch. In the 'wild' strain the peaks appeared at approximately every three days. During the first 10 days of egg

laying the variations rarely exceeded 5%. Thus in the rearing program where a large number of jars were being prepared each day adjustments had to be made in order to provide for these cyclic variations in the hatching of the eggs.

Survival of Larvae

After the rearing medium has been 'seeded' with eggs it is not possible to follow closely the behavior of the larvae without disturbing the balance of conditions within the rearing jars. Estimates of larval survival were based, therefore, on a count of the puparia produced and the percentage of hatched eggs. No precise method of obtaining data upon the time of greatest larval mortality was devised, although under normal conditions, it appeared that most of the mortality occurred soon after the eggs had hatched, when the young larvae were becoming established.

Differences in survival of larvae occurred in much the same manner as in the case of the eggs, i.e. from one population to another and in daily cycles. The variations in survival of the larvae, however, were greater. It was also found that when the percentage of hatched eggs was unusually high the survival of the larvae was appreciably lower and vice versa. Thus, when the percentage hatch of the eggs was high some of the larvae were weakened and soon died and the proportion of less viable larvae decreased as the percentage of hatched eggs decreased.

The Rearing Medium

During incubation of the larvae active fermentation occurs in the rearing medium resulting in an almost continual change of chemical substances and the production of heat. Exact control of all the variables is almost impossible. In the present study it was found that the production of puparia was dependent to a large degree on both the quantity and quality of fermentation particularly during the early stages of larval development, and that unless some form of control was used the rearing was very unpredictable. On the other hand, apart from the necessity of using fresh yeast (can be held at 0° to 1° C. not longer than 10 days), varying the concentrations of the different ingredients composing the rearing medium had little effect on production of puparia.

The process of fermentation was influenced almost exclusively by temperature, aeration, and the water content of the rearing medium. Moisture was subject to more or less exact control by altering the amount of water used when preparing the medium and its effect on fermentation, therefore, was largely indirect. If the medium became too wet, aeration was poor and fermentation dropped, whereas if the medium became dry aeration was excessive and fermentation proceeded too rapidly.

Temperature and aeration, however, were not subject to such precise control, since they were both influenced by the amount of fermentation. The temperature of the rearing medium was the resultant of the heat of fermentation

and the loss of heat to the incubation chamber. Aeration was dependent on the wetness of the rearing medium, the amount of packing or settling of the medium in the jars, and the activity of the developing larvae in crawling through the medium.

A study was made of the temperature characteristics of the rearing medium in the jars as an index of fermentation. Recordings made from thermocouple points placed at different levels in the medium gave a general picture of the thermal changes in the medium from the first day of rearing until the puparia were formed. The results obtained from jars seeded with 2500 eggs and at a depth of 3 in. below the surface are shown in Table IV.

TABLE IV

TEMPERATURE CHANGES IN BATTERY JARS OF REARING MEDIUM INCUBATED AT 26.7° C.

Days of rearing	Temperature in ° C.
One, just after seeding eggs	32
One, six hours later	35
Two	42
Three	45
Four	44
Five	43
Six	36
Seven	32
Eight	30
Nine	29

Although both vertical and horizontal temperature gradients were constantly present, except for those at the immediate surface and circumferential layers, they rarely exceeded 3° C. As can be seen in the table, the temperature of the medium increased until the third or fourth day of rearing after which it began to decrease. The maximum temperature in these lots was 45° C. This temperature appeared to be somewhat critical since above this range serious crowding of the larvae ensued. Developing larvae had a definite temperature preference of 40° to 42° C. and would, therefore, migrate to the lower temperatures at the peripheral and surface layers where crowding would result in excessive mortality and the formation of abnormal puparia.

When fermentation was poor and the temperature of the medium did not exceed 36° C. development was prolonged by two to three days and survival of the larvae and production of puparia was low. The medium also remained

wet and tended to pack into sticky masses making removal and separation of the puparia very difficult. The effects of various temperatures of the medium on puparial production are shown in Table V.

TABLE V

DIFFERENCES IN PUPARIAL PRODUCTION AT DIFFERENT TEMPERATURES OF THE REARING MEDIUM

Experimental number	Normal temperatures	Abnormal temperatures
Generation 18-3		
Highest recorded temperature	41° C.	47° C.
Number of puparia per jar	1650	1290
Survival of larvae, %	76.7	59.9
Generation 32-2		
Highest recorded temperature	41° C.	46° C.
Number of puparia per jar	1540	1510
Survival of larvae, %	78.6	77.0
Generation 25-3		
Highest recorded temperature	42° C.	36° C.
Number of puparia per jar	1750	1080
Survival of larvae, %	87.5	54.0

Various attempts were made to control the rate of fermentation in the battery jars and thus decrease the possibility of variation in production. Aeration of the rearing medium was regulated to some extent by avoiding packing when filling the jars and by seeding with a constant number of eggs. Variations in aeration were chiefly due therefore, to the differences in larval activity. During the first day or two of rearing, young larvae were evenly distributed throughout the medium. As the temperature of the center of the medium rose to 42° C. and above, the larvae migrated to the surface layer where their feeding and movements made the medium loose and finely divided, allowing access to the air. This normally occurred on the third day. As incubation continued, the larvae fed at greater depths bringing air to the mass of medium lower in the jar. Aeration could only be controlled, therefore, by preventing the formation of a crust of medium in the upper layer before the larvae had an opportunity to feed at the surface. This was accomplished by covering the jars with damp towels for at least the first three days of incubation. In battery jars, under normal conditions of rearing, control of the temperature was almost impossible and could only be attempted by regulating the room temperature within rather narrow limits of from 24° to 28° C.

To provide a more precise means of maintaining temperature control and thus decrease variations in puparial production, wooden boxes were used very effectively for rearing. These were fitted with copper tubing through which water was circulated at a constant temperature throughout the medium. Using these rearing units surprisingly uniform production was obtained. In an experiment designed to compare the production of puparia in medium at

constant temperatures 5° C. higher and 4° C. lower than the normal range of rearing as shown in Table IV, no significant differences in the number of puparia were observed, although at the higher temperatures a greater number of smaller puparia were formed. This is shown in Table VI.

TABLE VI

PUPARIAL PRODUCTION IN BOXES FITTED WITH TEMPERATURE-CONTROL COILS

	Puparia from medium equivalent to one battery jar	Survival of larvae, %	Weight of puparia (per 1000)
Mean at normal temperature	1650	84.7	19.258
Mean at 5° C. above	1518	78.3	17.561
Difference	132	6.4	1.696
Number of tests	5	5	5
P values	0.1	0.1	0.05
Mean at normal temperature	1554	85.3	17.930
Mean at 4° C. below	1568	86.2	18.122
Difference	−14	−0.9	−0.192
Number of tests	5	5	5
P values	>0.5	>0.5	>0.5

Density of Larval Population

The optimum density of larvae in the rearing jars was obtained by seeding them with 2500 eggs. At this density and under standard rearing conditions from 1600 to 1800 puparia were recovered. At higher densities the survival of larvae was much lower, particularly, as in many instances, where the temperature became abnormally high. Although it was found possible to rear flies at densities up to 7500 eggs per jar, survival of the larvae was very low and the production of puparia much reduced.

Using wooden boxes in which temperature of the medium was under constant control, a series of 10 tests was made to compare production of puparia at densities of 2500 and 5000 eggs per quantity of medium equal to one battery jar. In each test six boxes were seeded with 2500 eggs per jar of medium and in six others twice the number of eggs were used. The results are shown in Table VII.

TABLE VII

PRODUCTION OF FLY PUPARIA AT DIFFERENT DENSITIES OF EGGS

	Number of puparia per jar of medium	Survival of larvae, %	Weight of puparia per 1000
2500 eggs per jar of medium	1795	88.4	18.395
5000 eggs per jar of medium	3165	78.2	14.139
Mean difference	−1370	10.2	4.256
Number of observations	10	10	10
P value	<0.01	<0.01	<0.01

From the statistics shown in the table it is clear that crowding of the developing larvae has marked effects on the production of puparia. This is expressed in a reduction of the number of puparia produced from each egg (from 72 to 63%) and/or as an increase in the mortality of the larvae and a 25% decrease in the size (weight) of the puparia.

Differences Between Lines of Breeding Stock

Differences in the production of puparia were apparent not only between different populations but between different lines of breeding stock. The constantly high production of certain selected lines was of considerable value in the production of a uniform and predictable quantity of puparia.

Early in the studies a series of selection experiments was carried out beginning with the 18th generation of laboratory reared flies. A number of pure bred lines were established, in which selection was maintained on the basis of puparial production per battery jar of medium. The number of eggs used was the same throughout. Selection was continued until the 30th generation. At that time no further improvement was evident and the most productive line was used to replace the general breeding stock. A summary of puparial production for five of the selected lines and the general laboratory stock is given in Table VIII. The statistics shown represent the mean values calculated from at least 10 daily lots from each of the generations indicated under the different lines.

TABLE VIII

PUPARIAL PRODUCTION FROM DIFFERENT LINES OF BREEDING STOCK

	Puparia per jar		Survival of larvae, %		Weight of puparia (per 1000)		Number of generations
	Mean	σ	Mean	σ	Mean	σ	
General laboratory stock	1600	300	82.8	12.5	19.32	1.50	47
Line *A*	1750	255	87.2	10.3	18.58	1.50	13
Line *B*	1430	237	76.9	11.9	20.00	0.87	7
Line *C*	1440	254	78.6	13.5	20.14	0.92	11
Line *D*	1820	154	93.1	6.0	19.51	1.14	7
Line *E*	1130	332	62.7	13.3	18.76	0.76	2

From a comparison of the lines, both *A* and *D* gave significantly greater production than any of the others and Line *D* was the most productive of all with respect to the number of puparia and the survival of the larvae. It is also noticeable that the variance in the *D* line was much lower than in any of the others. The differences in size of puparia were not significant.

Using only flies from the *D* line as breeding stock large scale production of puparia was carried out for two years in a very uniform and predictable

manner. From the production records through 88 generations and on occasions at the rate of almost one million puparia per day the following constants were obtained.

Mean number of puparia per jar of medium	1770 ± 240
Mean survival of eggs placed in jars, %	70.9 ± 9.3
Mean percentage of eggs that hatched	80.1 ± 5.1
Mean survival of larvae, %	87.5 ± 8.6

In obtaining these constants, calculations were based on propagation records from only the first 10 days of egg deposition and under rearing conditions to be described later in which 2500 eggs were used per battery jar of rearing medium.

Differences in Laboratory Populations of Flies

Populations of flies when used as breeding stock or as test animals in experimental studies showed considerable variation with respect particularly to the percentage of adult emergence from the puparia, the sex ratio, length of adult life, fecundity, and the periods required for adult emergence and egg deposition. The variations in length of life and fecundity were greatest, although the influence of all had to be considered in the routine rearing and testing of the flies.

Adult Emergence

When puparia were incubated immediately following separation from the rearing medium at 26.7° C. and 50% relative humidity little variation in adult emergence occurred. Under these conditions the first adults appeared in 3 ± 0.5 days and nearly all the puparia produced normal flies. The mean adult emergence from puparia reared through 40 generations was 92.6 ± 2.4%. If, however, the period between the formation of the puparia and emergence of the adults was prolonged particularly by lower temperatures both the number and rate of adults emerging was considerably altered and became very variable. A detailed account of experiments with puparia at low temperatures is given in a later paper of this series.

Sex Ratio

Although the sex ratio of the emergents varied somewhat from one group of flies to another in most samples containing over 10,000 it was remarkably constant. The mean sex ratio (percentage of females) of flies reared in 40 generations, representing over one million individuals, was 50.0 ± 5.0. In small samples the sex ratio varied from a minimum of 40 to a maximum of 60% females depending on when the sample was taken. Since males normally emerge before the females, samples taken early in adult life will contain a preponderance of males, while if the sample is taken after three days of adult life there will be a greater proportion of females, since males have a much shorter life than females.

Length of Adult Life

Considerable variation in length of adult life was noticed between different populations of flies. Individual differences within a population were even more striking. Males began to die three to four days after emergence. In breeding cages a few males were dead on the first day of oviposition and most of the males had died by the 10th day of laying, although some lived for as long as 20 days. Death of the females began on about the third day of egg laying (six to seven days old), about one-third being dead after 10 days of oviposition. A very small proportion of the females lived for a maximum of 40 days. The mean length of life of flies taken from generations 20 to 60 was, for males 12 $\pm$ 2 days and for females 20 $\pm$ 2 days. The percentage dead in the breeding cages after 10 days of oviposition was, for males, 71.1 $\pm$ 19.3 and for females, 31.6 $\pm$ 13.0.

In order to obtain a constant and predictable supply of eggs, care had to be exercised in guarding against excessive female mortality. This was done by keeping the breeding cages liberally supplied with dishes of skimmed milk and water. Attempts were made to prolong the life of flies by feeding them whole milk at various dilutions as well as milk with malt and yeast, or both, but there were no significant differences either in mortality or fecundity.

Fecundity

The greatest variable encountered in rearing the flies was in the number of eggs deposited by the females. In determining the number of flies required for the propagation of a predictable number of puparia certain empirical corrections always had to be made. Experiments in which individual flies were used showed that many of them laid no eggs. It was found, however, that isolated flies rarely produced a normal number of eggs and few laid more than 200. In larger groups of adults, egg production for cages of 500 flies (the average for 40 generations) was 500 $\pm$ 100 per female, 65% of which were laid in the first 10 days of oviposition. The rate of oviposition was 30 $\pm$ 10 per female per day for the first 10 days. After that time egg deposition decreased rapidly and at an irregular rate.

In all populations of flies the greatest production of eggs occurred on the second or third day of oviposition, and decreased as the flies aged. Even before the 10th day oviposition was characterized by having daily fluctuations, a day of high egg deposition being followed by one of low deposition and vice versa. Some of these variations were caused by differences in the mortality of females and by the escape of females from the cages when food dishes were changed. Even under carefully controlled experimental conditions appreciable variation occurred. A number of possible causes were investigated, including size of the adult, type of food, the temperature and population density at which the flies were reared, the days on which eggs were laid, and selection of breeding lines. No significant correlations were obtained from the data secured in 53 replicate samples through 13 complete generations.

Time of Emergence and Oviposition

Differences in the time between removal of the puparia from the rearing jars, adult emergence, and subsequent egg deposition depended almost entirely on the stage of puparial development at the time the puparia were separated from the medium. When rearing was done in battery jars the puparia were removed on the morning of the ninth day, i.e. after eight complete days of incubation. At 26.7° C. 140 hr. were required between the formation of puparia and adult emergence. Females required approximately three hours longer than males. Puparia, when removed from the rearing jars had usually completed an average of about 70 hr. of development and approximately half the puparia had undergone 65 to 95 hr. of development. Thus the amount of development, being a function of the temperature of the rearing medium, had a marked influence on the time of emergence and oviposition.

In wooden boxes where the temperature of the medium could be accurately controlled, differences in the age of the adult stock within comparable populations were very much reduced. Under these conditions puparia were removed after seven complete days of rearing. At that time the mean age of the puparia was 70 hr., but over 80% were within the age class of 65 to 95 hr. of development and over 80% of the adults would emerge within a 24 hr. period.

Differences Between Wild and Powell Strains

For the most part, in the experiments carried out during the present study the flies were propagated from a selectively-reared 'Powell' strain used almost exclusively in Peet-Grady tests. In order to compare the results with those from wild populations 40 generations of flies reared from a stock of adults captured near Belleville were propagated in a similar manner and under the same incubation conditions.

From the records of production and adult behavior it was found that the wild strain had variations similar in almost every respect to the Powell strain and that the differences between the two strains were very slight. No significant differences were found in the percentage of adult emergence, sex ratio, rate of development and oviposition, or in fecundity. Although it was noticeably more difficult to induce the wild females to lay their eggs and in most cases it was necessary to mix a little of the rearing medium with the milk in the oviposition dishes, the total number of eggs deposited was essentially the same. The wild flies lived a little longer than the Powell flies, the mean length of life of the females being four days longer; the life of the males was of similar duration. The greater mean duration of life of the wild flies was due largely to the presence of a greater proportion of individuals that lived far past the normal life span. The maximum longevity of wild flies in cages was 50 days.

Under favorable conditions of rearing, particularly with respect to fermentation, greater larval mortality occurred in the wild strain. It appeared that the adaptability of larvae from the wild strain was less than larvae from the

Powell strain and that under unsuitable conditions the Powell stock was more productive of puparia. A greater number (about 3%) of the eggs deposited by the wild strain produced larvae but fewer of the Powell larvae died during rearing so the production of puparia was the same.

Methods Adopted for Large Scale Production

For the handling and propagation of the large quantities of flies required, the technique of rearing outlined under general methods of rearing (p. 9) was completely inadequate. By following the usual technique the space and time required for rearing were far beyond the scope of available facilities and, in addition, were a source of considerable variation with respect to both production and the behavior of the flies during tests. Studies were, therefore, undertaken with a view to devising more suitable methods of propagation. After considerable experimentation a procedure was finally adopted that eliminated most of the undesirable features of the previous method and yet made it possible to rear on a predictable and continuous basis millions of flies per day. An outline of the methods used is given under the headings listed below.

Handling Adult Flies During Mating and Egg Deposition

The cages used to hold the flies for mating and oviposition were made up as required from demountable wooden units fastened together to form a rectangular structure 3 × 3 × 3 ft. Each of the four sides as well as the top and bottom consisted of a screen covered section that, when assembled, could be fastened to its neighbors by carriage bolts and wing nuts. Thus the cages could be readily dismantled for cleaning. Across one side were three rows of paired wooden doors, permitting access to the cage. The edges of the doors were lined with weatherstripping to prevent escape of the flies. From the bottom of each door to the opposite side of the cage were placed two movable wooden strips separated by a distance equal to the width of a cafeteria tray. The trays, of enamelled metal 17 × 12 × $\frac{3}{4}$ in. had an overhanging edge by which they were held in place on the wooden strips. Six trays were used in each cage.

Before being placed into the cages the trays were filled with a milk solution consisting of equal parts of skimmed milk and water. Each tray held about one pint of milk solution. The surface of the fluid was covered with paper towelling supported on small blocks of cork and a small crumpled piece of towelling laid on top. Most of the eggs were deposited upon the relatively drier surface of the upper piece of towel. Cages set up in this manner provided approximately 70 sq. ft. of roosting surface and would satisfactorily accommodate from 10,000 to 20,000 flies.

Each morning the trays of milk were removed and replaced by fresh trays. The eggs were washed from the towelling into a jar of water, using a fine spray nozzle. The egg suspension was passed through a 14 mesh screen to remove dead adults and large milk clots and then into an 80 mesh screen container

where the eggs were washed free of milk particles with a fine spray. The eggs were later resuspended in water and poured into a glass tube $\frac{1}{2}$ in. in diameter, capped on the bottom with a removable piece of 80 mesh screening. The number of eggs could be estimated within a maximum error of $\pm$ 4% by a calibration of the tube. One inch of the tube held 16,000 eggs, provided at least a 2 in. head of water was maintained in the tube above the eggs.

From each of 15 populations of 10,000 flies handled in the above manner, two samples of 500 flies were withdrawn and treated according to the usual Peet-Grady methods. Counts of egg deposition and hatch were made on this series of paired populations and the results analyzed by the 't' test for paired variates. A small but significant difference was found in the hatching of the eggs; 84.5% of the eggs from the small cages produced living larvae, while only 80.4% of the eggs hatched from the large cages. Apparently a greater proportion (4%) of eggs was injured during cleaning and measuring from the larger cages. Differences in fecundity were very small and in no cases were they statistically significant.

Larval Rearing Containers

A number of different types of rearing containers were constructed and tested in large scale puparial production. For moderately large propagation (from 100,000 to 300,000 puparia per day) the best larval rearing container was a wooden box of the dimensions 16 $\times$ 11$\frac{5}{8}$ $\times$ 6$\frac{1}{2}$ in., constructed of $\frac{7}{8}$ in. cypress or cedar in which could be placed the equivalent to four battery jars of rearing medium. The box was sealed on the inside with a coating of hard paraffin and beeswax. Each box was fitted with a set of copper coils by which the temperature of the medium was constantly controlled. The coils were made up from $\frac{5}{8}$ in. copper tubing 5 ft. long, bent into a circular pattern and placed on small blocks one inch from the bottom of the boxes. The coils were joined to a water conduit system equipped with hand operated valves, a centrifugal pump, and a reservoir in which the water was maintained at a constant temperature of 36° C.

The use of the water circulating system provided an excellent means of controlling fermentation and it was thus possible to greatly reduce variations between different units or lots. This was of particular value in reducing the spread in ages of the puparia and in eliminating excessive larval mortality associated with abnormally high temperatures. Production of puparia was as high as in the battery jars. The mean number of puparia produced per box seeded with 10,000 eggs during 40 generations of continuous propagation was 7080.

A number of other types of rearing containers were tried with varying degrees of success. Of the different types used, biscuit tins holding four battery jars of medium, galvanized iron trays holding 60 jars of medium, and wooden trays holding 144 jars were the most satisfactory. In general, as the

size of the rearing container was increased, the mortality of the larvae increased and the puparia produced were smaller, owing to the development of high temperatures in the larger masses of medium. In metal containers, where the heat accompanying fermentation was dissipated rapidly, puparial production was equivalent to that in battery jars but the metal corroded very rapidly and soon had to be replaced. In the large wooden trays temperatures rose as high as 60° C. and it was, therefore, essential to equip them with a cooling device made up from copper pipe operating through a pump and water conduit system. By regulating the temperature and flow of the water and by limiting the depth of the medium to 6 in., conditions within the medium could be controlled sufficiently to produce puparia comparable in number and size to those from battery jars and the small wooden trays. The average production of puparia per large wooden tray was 250,000.

Rearing Medium

In order to avoid the laborious task of mixing by hand a machine was constructed to handle the greater quantities of medium required in large scale rearing. The mixer was made up by mounting a 50 gal. oak barrel on a horizontal axle and was revolved by means of a $\frac{1}{4}$ h.p. electric motor and a series of differential pulleys. A door 9.7 in. wide made by removing several staves along one side was held in place by heavy spring clamps and fitted tightly against a rubber gasket. Up to 48 jars of medium could be mixed at one time.

For the most part, the medium used for rearing larvae was mixed 24 hr. before being seeded with eggs. Medium stored at room temperature for two days in battery jars was satisfactory for rearing. When the large temperature-controlled boxes were used, freshly prepared medium seemed to give best results.

During most of the rearing the modified Peet-Grady formula outlined on p. 9 was used in making up the medium. At times during the course of the work, alfalfa meal could not be found in sufficient quantity and it was necessary to try substitutes. After experimenting with a number of substances it was found that the larvae could be reared in a medium in which all or part of the alfalfa meal was replaced by sawdust. Medium sized sawdust gave best results. Sawdust could not be substituted for any part of the bran.

The use of sawdust as a substitute for alfalfa in the rearing medium had a marked effect on the production of flies. The yield of puparia was considerably lower and there was a noticeable reduction in the emergence of adults from the puparia. The adults that did emerge, however, although somewhat smaller in size, reacted the same in experimental tests as individuals reared on normal medium. In Table IX a comparison is made between paired populations of flies reared on standard sawdust media. The differences are statistically significant in the first five columns and not significant in the last two.

TABLE IX

DIFFERENCES BETWEEN FLIES REARED ON STANDARD MEDIUM AND MEDIUM IN WHICH SAWDUST WAS SUBSTITUTED FOR ALFALFA MEAL

	Puparia per jar of medium	Survival of larvae, %	Survival of eggs, %	Weight of puparia (1000)	Emergence of adults from puparia	Eggs per female per day	Adult mortality in first 10 days, % of total
Standard medium	1592	80.8	63.6	19.41	93.0	23.0	54.3
Sawdust medium	1402	70.3	56.1	14.35	81.6	22.9	59.1
Mean differences	190	10.5	7.5	5.06	11.4	0.1	−4.8
Number of observations	5	5	5	5	10	5	5
P value	0.03	0.04	0.03	0.02	<0.01	>0.5	0.2

Separation and Treatment of Puparia

To dry and separate the large number of puparia from the medium a wooden-framed, masonite tower 2 × 2 × 6 ft. was found to be very useful. Twelve wooden-framed, screen bottom trays, 3 in. high, were placed in the tower and the air drawn through them by means of a high speed exhaust fan mounted on the top. Each screen tray held the puparia and upper layer of four jars of medium. The contents of 12 trays (100,000 puparia) was dry and ready for separation at the end of an hour.

Separation of the puparia from the dried medium was accomplished in a very short time by passing the mixture through an electrically-driven grain seed or bean cleaner. Using this winnowing machine 500,000 puparia could be thoroughly cleaned and separated from all waste in one hour.

Separation of the puparia is also possible by repeated washing in water. The puparia float on the surface of the water and can be readily skimmed off the top. These puparia are particularly free from adhering waste particles and require no previous drying.

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STUDIES ON THE HOUSEFLY (*MUSCA DOMESTICA* L.)

II. THE EFFECTS OF LOW TEMPERATURES ON LABORATORY-REARED PUPARIA[1]

By G. E. Bucher,[2] J. W. MacB. Cameron,[3] and A. Wilkes[4]

Abstract

Investigations are reported on factors influencing the survival of housefly (*Musca domestica* L.) puparia exposed to different intensities of low temperature storage above 1° C. Survival was decreased by lowering the temperature of storage and by increasing the duration of the storage period, or both. Mortality of adults following puparial storage was influenced by the type of rearing container used, crowding, age of puparia, humidity, concentration of gases in the pupal rearing chambers, and to some extent by changes in the food of the immature larvae but not by the age or sex ratio of the parental stock, the size of puparia, selective breeding of resistant individuals or different strains of stock. Death did not occur in cold storage but during subsequent incubation at normal temperatures and at a definite stage in development near adult emergence. Temperatures below the threshold of development caused physiological disturbances that affected the longevity, oviposition, and hatchability of eggs of the adults that survived. A proposed explanation is given of the lethal effects of low temperatures based on the interrelationships of disturbances between the relative rates of development and differentiation of various ontogenetic systems.

Introduction

The importance of temperature as a factor governing the lives of insects and other poikilothermic animals is demonstrated by the vast literature, surveys of which have been made by Payne (12), Uvarov (21), and Bělehrádek (3). Entomologists have been concerned chiefly with the influence of temperature upon the rate of metabolism or the speed of development and with considerations of the factors by which insects are able to survive periods of extreme cold.

Although a number of authors observed that the time of exposure might affect the survival of cold-hardy insects in freezing temperatures, little attention has been paid to a general study of the "quantity factor" referred to by Payne (13). Studies of the effect of prolonged exposure to temperatures at which protoplasm does not freeze have been confined to the insects of stored grain (*vide* Back and Cotton (1); Larson and Simmons (10); Carter (6); Robinson (17)). There is consequently little information available on the effects of prolonged exposure to moderately low temperatures and there is no universal agreement as to the cause of death in insects that are not resistant to this treatment.

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The present investigation upon the resistance of housefly puparia to cold and the physical and biological factors that influence survival was undertaken to determine the possibility of building up large populations by continuous propagation and low temperature storage. Although the experimental evidence demonstrated that this was not practical and cast doubt on the ability of houseflies to overwinter as puparia, the data obtained are of value in providing further information on the reactions of common insects to the so-called quantity factor. The experimental evidence presented in the present study supports the theory of development suggested by Powsner (16), as an explanation of the lethal action of temperatures above the freezing point of protoplasm.

This paper was taken from a dissertation presented by the senior author (Bucher) in partial fulfillment of the degree of Doctor of Philosophy at the Ohio State University. For tables of original data and a more detailed statistical analysis, reference may be made to the dissertation deposited in the library of the Ohio State University.

Methods of Experimentation and Analysis of Results

The fly puparia used in the experiments were reared by a modification of the Peet-Grady method, an outline of which is given in Part I of this series.* Samples of 500 puparia were withdrawn at random from well-mixed populations, placed in storage containers and exposed to various periods of low temperature. At the conclusion of the storage treatment, the puparia were incubated at 27.6° C. until emergence of the adults had ceased. The adults that emerged were counted, checked for errors by a count of unemerged puparia, and the number expressed as percentage emergence or percentage survival.

The resistance of a puparium to cold storage was, therefore, measured by an all-or-none criterion, the emergence or nonemergence of an adult fly after return to the incubation temperature. Individual variations in resistance occurred between puparia of a single population, reared and stored in an identical manner, so that, after any given treatment, part of the population succumbed while the remainder survived and produced adults. By the use of a sample of puparia and the calculation of percentage survival, a measure of the resistance of puparia to storage treatment was obtained. If samples were withdrawn from a population of puparia and subjected to different intensities of storage, or if samples were withdrawn from different populations and subjected to the same intensity of storage, differences in survival were obtained. It was therefore necessary to determine what proportion of these differences in survival was due to experimental treatment or to

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innate differences between populations, and what proportion was due to chance, in order that the significance of variations in survival of samples could be assessed.

It was found by experiment that, if a series of samples of 500 puparia were withdrawn from a well-mixed population and subjected to identical storage conditions, the percentage survival of any one sample rarely varied from the mean of the series by more than 2.5, while the difference between the highest and lowest samples rarely exceeded 5.0. This result conformed closely to that predicted by the law of random variation of percentages in which $s = \sqrt{\frac{p.q}{n}}$, where p is the population parameter (i.e. in this case the true survival of the population), q is 100-p, n is the number of individuals in the sample, and s is the standard deviation of the statistic (in this case the expected deviation in percentage survival between samples caused by chance alone). If p equals 50%, for a sample of 500, s becomes 2.2%. That is, within a series of samples treated alike, about two-thirds of the samples would show survivals within 2.2% of the mean and be within a range of 4.4%.

In the experiments two samples of puparia were used for each treatment and the percentage survival calculated for each separately. When the survivals varied from each other by 5% or less, the mean was accepted as representing the best available measure of the population parameter. If the survivals varied by more than 5% (a rare occurrence), the records were discarded as not reliable. The mean survival figures for a given treatment and population are considered as accurate to within 2.5%. Theoretically, as the population parameter approaches 100% or 0%, the standard deviation decreases and smaller differences may be regarded as significant. In these studies, however, a difference of less than 2.5% was regarded as not significant at any value of the parameter.

The validity of the sampling technique and the use of the equation $s = \sqrt{\frac{p.q}{n}}$ to estimate the standard deviation due to chance was based upon the hypothesis that the fly population was normally distributed and that no large components of the population were inherently more resistant than others and thus predisposed to survival. In order to test this hypothesis and to determine whether resistance to cold was inherited, a series of selection experiments was carried out.

A population of 70,000 puparia was stored at 6.0° C. for 15 days. The surviving adults, numbering about 1%, were bred and their progeny reared, the puparia being stored at 6.0° C. for 10 days. Surviving adults were again bred and the puparia from the filial generation stored at 6.0° C., for 10 days. This procedure was repeated for seven generations.

Survival of puparia did not increase as the selection was continued. After seven generations survival of the selected population was compared with a population of equal age from the normal breeding stock (Table I). There was no significant difference in survival.

TABLE I

SURVIVAL OF PUPARIA FROM SELECTED AND NORMAL POPULATIONS AT 9.4° C.

Days of storage	Percentage survival of puparia	
	Selected strain (mean age 68 hr.)	Normal strain (mean age 70 hr.)
0	93.2	94.2
3	93.6	92.8
5	87.2	90.8
10	70.0	69.8

Selective breeding of the portion of a population surviving puparial storage over seven generations did not increase the resistance of puparia to storage. Thus it is concluded that selection did not produce a change in cold resistance.

Puparia from some populations displayed considerably more resistance to cold than those from other populations. The most important of the factors causing these differences in resistance were the temperature of rearing and the age of the puparia when stored. In order to obtain reliable data from which to determine the amount of resistance of puparia subjected to various storage treatments, it was necessary to reduce variations between populations caused by differences in age, temperature of rearing, and other factors, and devise a rearing technique whereby puparia of maximum and uniform resistance could be produced in numbers. Puparia reared in this manner were designated as 'normal'. Normal puparia in this case were those reared at a density of 2500 eggs per jar unit of standard rearing medium in temperature-controlled boxes or in battery jars in which the temperature cycle followed a normal pattern and the maximum temperature did not exceed 42° to 44° C.

At the commencement of storage treatment using normal puparia about 80% were within the most resistant age group, while nearly all were within the range of 40 to 110 hr. The mean age of any experimental population was estimated by dissection or calculated from time-emergence records of untreated samples.

A small number of newly-formed puparia or puparia close to emergence was present occasionally even in a normal population. When experimental samples were withdrawn these were recognizable by color and rejected, as well as puparia with injuries or other abnormalties. The number of rejected puparia never exceeded 1%. With these exceptions, samples were withdrawn at random from well-mixed populations.

Temperatures of 1.0°, 6.0°, and 26.7° C. were secured in temperature-controlled chambers, having an accuracy of ± 0.5° C. Other temperatures were obtained in a multiple constant-temperature cabinet that operated at an accuracy of ± 0.1° C.

The flies used for the most part in these studies were reared from the Powell stock. A wild strain was propagated from flies collected at Belleville. Parallel

tests, conducted on both strains, indicated that there was no significant difference in their resistance to low temperatures. Therefore, while the data here reported are based upon a strain of flies of considerable genetic homogeneity, the results are equally applicable to wild flies.

The Influence of Age of Puparia on Survival

It has long been recognized that age has an important influence upon the resistance of both plants and animals to chilling. In insects, some developmental stages are more resistant than others (Bodine (5); Ludwig (11)). Within any given developmental stage, age may also have an effect on resistance as shown for ant pupae by Pictet (15). In the housefly, where the pupal stage is relatively short and apparently includes no diapause, an enormous amount of metabolic activity must occur. It was likely, therefore, that at certain periods in metabolism, the organism would be more susceptible to unfavorable temperatures than at others. To test this hypothesis, puparia of known age were subjected to a period of five days' storage at 6.0° C.

The results obtained from the series of experiments are shown in Table II. Age was measured in hours at 26.7° C.

TABLE II

VARIATION IN RESISTANCE TO COLD STORAGE WITH AGE OF PUPARIA (FIVE DAYS AT 6° C.)

Limits of age of puparia when stored	Mean age of puparia	Sample size	Emergence (survival), %
0- 1 hr.	0 hr.	397	12.6
1- 2	1	136	1.5
2- 3	2	123	14.6
3- 4	3	299	23.1
4- 5	4	293	31.1
5- 7	6	182	13.2
7- 9	8	268	11.6
9- 11	10	282	10.3
11- 13	12	247	9.3
13- 15	14	178	8.4
15- 17	16	219	0.9
17- 19	18	144	0.0
19- 21	20	207	5.3
21- 23	22	733	16.6
23- 25	24	768	34.8
25- 35	30	2098	64.2
35- 45	40	1851	66.4
45- 55	50	1657	73.9
55- 65	60	1299	77.1
65- 75	70	1140	82.1
75- 85	80	885	79.6
85- 95	90	1293	83.3
95-115	105	825	74.7
115-135	125	833	73.5
135-140	Emergence begins		
Control	No storage treatment	762	85.4
Mean emergence 65-95 hr.			81.7

Puparia less than 30 hr. of age at 26.7° C. were very susceptible to killing in cold storage. Resistance increased slowly with age above 30 hr., reached a maximum at 90 hr., and thereafter decreased slowly. At 26.7° C., between 135 to 140 hr. of development were necessary for pupation, females usually requiring one to two hours longer than males. The curve of resistance as influenced by puparial age is shown in Fig. 1.

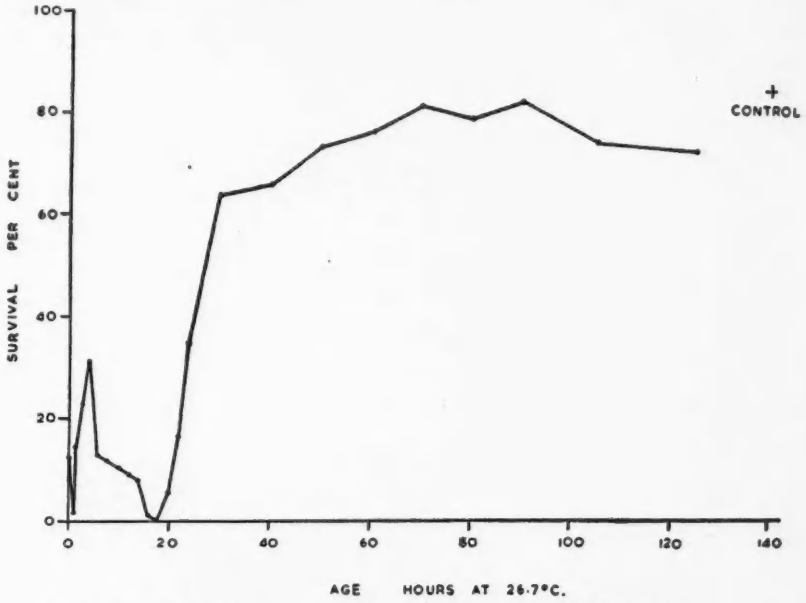

FIG. 1. *The survival of puparia of different ages exposed to 6° C. for five days.*

Since puparia were most resistant to cold temperatures when stored after 65 to 95 hr. of development at 26.7° C., attempts were made to use, for further experiment, populations in which the majority of the puparia were within this age group. In order that the age of the population could be estimated before experimenting, a brief study of the most evident morphological changes during pupal development was undertaken.

Between 0 and 12 hr. of development at 26.7° C. puparia change from cream to buff to red, and thus age can be recognized by inspection. The interior is wet and superficially disorganized. The head region is larval in appearance, is fastened to the puparial wall, and will pull off with the first segment. The mouth hooks are intact and there are tracheae attached to the anterior spiracles.

Between 12 and 24 hr. the head becomes more clearly defined, the antennal areas being plainly visible.

After 48 hr. the body begins to assume an adult shape and is largely free from the puparial wall. The mouth hooks are free of the head and can be

found in the anterior portion of the puparial case. Tracheal connections to the anterior spiracles of the puparium have disappeared. The antennal areas become buff colored. Leg and wing buds can be seen.

At 60 to 70 hr. body formation is more like the adult. The antennal areas become brownish in color.

Between 70 and 95 hr., the head assumes a definite adult shape. Compound eyes are distinct. They are white in color but gradually turn to amber at the close of the period. The ocellar spots begin to darken.

Between 95 and 120 hr. the compound eyes become rusty brown. The body begins to harden and darken. The appendages are well formed. The legs become fuscous.

Between 120 and 135 hr. the puparia turn black and the compound eyes red. The insect has the form and coloration of an adult.

By use of these rough morphological characters, the mean age or stage of development could be determined by dissection of a sample of the population. The most resistant stage occurred when the puparia had brownish antennal areas and distinct compound eyes, either white or amber in color.

Survival of Puparia at Different Intensities of Cold Storage

Variation in the intensity of low temperature treatment may be secured by varying either the storage temperature or the duration of the storage treatment. During the course of this study experiments were carried out to determine survival under different intensities of storage.

After the storage treatment, the samples of puparia were placed in open Petri dishes within screened cages at 26.7° C. until emergence had been completed, at which time survival was assessed.

When survival was plotted against the period in storage, a series of curves was obtained, each curve representing a different temperature of storage. It can be seen from the slope of these curves (dotted lines in Fig. 2) that about 10% of the puparia are considerably more resistant to storage than the remainder. Since the mean emergence, calculated from a large series of observations, was $92.6 \pm 2.4\%$ for unstored puparia it also can be seen that approximately another 10% of a normal population of puparia are extremely sensitive. Between these two extremes they exhibit varying degrees of resistance to cold storage. The relationship between percentage survival and period of storage appears to be parabolic in form, when the very resistant and very susceptible components of the population are disregarded.

In order to demonstrate the relationship between survival and the period of storage and to make an estimate of survival for any particular period of storage, equations were fitted to the data by the method of multiple regression described by Snedecor (20).

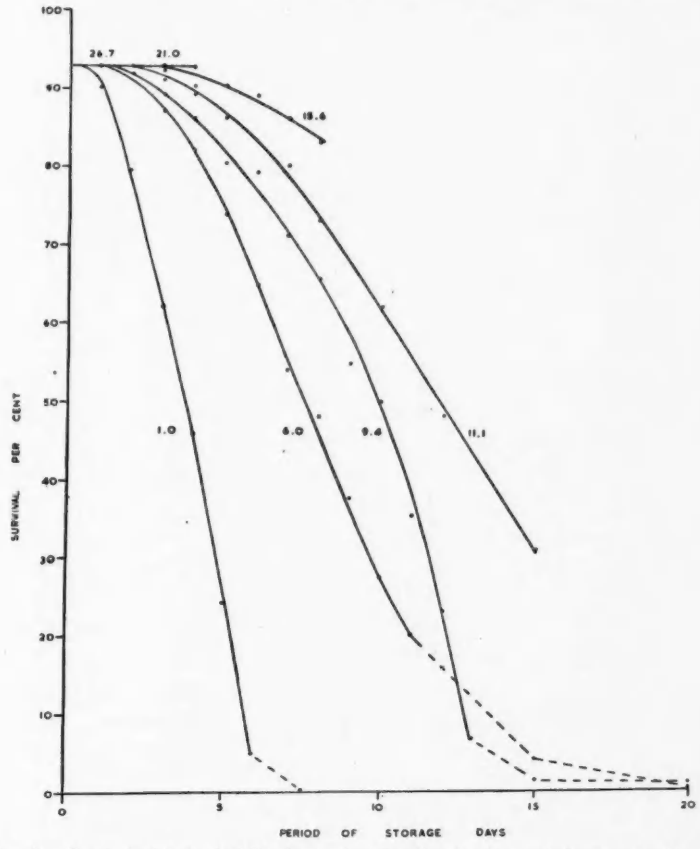

FIG. 2. *Survival of puparia following various lengths of exposure to low temperatures.*

Good fits were secured by the use of the polynomials of the form $Y = a + bX + cX^2 + dX^3$, where Y is the percentage survival, X the period of storage in days, and a, b, c, and d are constants specific for the temperature of storage. The equations are listed below.

Temperature	Equation	R
1.0° C.	$Y = 102.16 - 9.6697X - 1.465X^2 + 0.00335X^3$	0.99885
6.0° C.	$Y = 90.90 + 3.4660X - 1.7510X^2 + 0.07734X^3$	0.99745
9.4° C.	$Y = 98.09 - 4.3722X + 0.42165X^2 - 0.04802X^3$	0.97407
11.1° C.	$Y = 90.29 + 2.4913X - 0.74759X^2 + 0.02112X^3$	0.99941
15.6° C.	$Y = 90.29 + 2.3221X - 0.62924X^2 + 0.02935X^3$	0.98142

The above equations fit the data only for that proportion of the population that is not abnormally susceptible or resistant to cold storage, that is, for the

period of storage from one day up to and including the sixth day at 1.0° C., the 11th day at 6.0° C., and the 12th day at 9.0° C. Since at 11.1° C. and above, some development occurs, the storage period was limited by the beginning of adult emergence. Data for 11.1° C., therefore, end at the 15th day, for 15.6° C. at the 8th day, and for 21.0° C. at the 4th day.

Since there is no significant difference between emergence from puparia incubated at 26.7° C. and those stored at 21.0° C. or incubated at 21.0° C. until emergence was complete, for temperatures between 21.0° C. and 26.7°C., the equation for survival becomes $Y = a$ where a equals 92.6. Incubation at 28.0° C. did not reduce emergence. The effects of temperatures higher than 28° C. were not investigated.

Goodness of fit of each equation was tested by calculating the multiple correlation coefficient (R), which is a measure of the correlation between the experimental values of survival and those estimated from the equation. Perfect agreement would result in a value of 1.0000 for R. Since the calculated values of R were close to unity, it was concluded that the equations were good representations of the dependence of survival upon period of storage.

The equations were used to calculate the estimated survival of populations of puparia at different intensities of storage. The points in Fig. 2 represent the observed mean survival calculated from the original experimental data for a large number of different populations.

Using the estimated survival figures, obtained from the equations, a series of curves was drawn to show the dependence of survival upon the temperature for different periods of storage. The equations of these curves were of the form $S = a + b \log T$, where S is the percentage survival, T the temperature of storage (degrees C), and a and b are constants, specific for the period of storage. The equations obtained were as follows:

Period of storage	Equation
1 day	$S = 91.30 + 1.800 \log T$
2 days	$S = 80.00 + 14.780 \log T$
3	$S = 66.85 + 21.988 \log T$
4	$S = 54.00 + 30.200 \log T$
5	$S = 43.59 + 39.358 \log T$
6	$S = 22.72 + 55.640 \log T$
7	$S = -0.16 + 73.269 \log T$
8	$S = 23.87 + 91.226 \log T$
9	$S = 48.26 + 109.436 \log T$
10	$S = 72.14 + 126.940 \log T$
12	$S = 120.00 + 161.000 \log T$
General	$S = a + b \log T$

Plots of the above constants a and b against the time in days showed that they varied with respect to the storage period, according to polynomial regressions similar to those obtained with the original survival period data.

The equations for these regression curves were calculated to be as follows, where X is the storage period in days:

$$a = 93.0788 - 2.00920X - 2.05301X^2 + 0.061187X^3 \quad R = 0.99896$$
$$b = 2.3672 + 1.09518X + 1.68248X^2 - 0.055504X^3 \quad R = 0.99958$$

When the above expressions were substituted for a and b in the general survival temperature equation $S = a + b \log T$, an equation for survival in terms of the two variables, temperature and period of storage, was obtained as follows:

$$S = 93.0788 - 2.00920X - 2.05301X^2 + 0.061187X^3 + \log T$$
$$(2.3672 + 1.09518X + 1.68248X^2 - 0.055504X^3)$$

In Table III, survival, calculated from the above equation, is shown for the experimental temperatures for periods up to 15 days.

TABLE III

CALCULATED VALUES FOR SURVIVAL OF PUPARIA INCUBATED AFTER COLD STORAGE IN OPEN CONTAINERS

Period of storage (days)	Temperature of storage (° C.)				
	1.0	6.0	9.4	11.1	15.6
1	89.1	92.0	94.0	94.4	95.1
2	81.3	89.8	91.9	92.7	94.3
3	70.2	85.2	89.0	90.4	93.2
4	56.1	79.5	85.4	87.6	92.0
5	39.3	72.3	81.2	84.3	90.6
6	20.3	65.1	76.3	80.5	89.0
7		56.6	70.9	76.2	87.0
8		47.3	64.9	71.4	84.8
9		37.4	58.5	66.3	
10		27.0	51.6	60.7	
11		16.2	44.2	54.7	
12		5.0	36.6	48.3	
13			28.6	41.5	
14			20.4	34.5	
15			11.9	27.1	

Survival of flies from puparia exposed to low temperatures may be illustrated best by reference to the two factors of intensity, temperature, and duration shown on a curved surface. The isometric projection of the surface is shown in Fig. 3.

From these experiments it is clear that survival of housefly puparia in low temperature storage is dependent upon both the temperature and duration of the storage period. For this species survival decreases as the temperature decreases and as the period of storage increases.

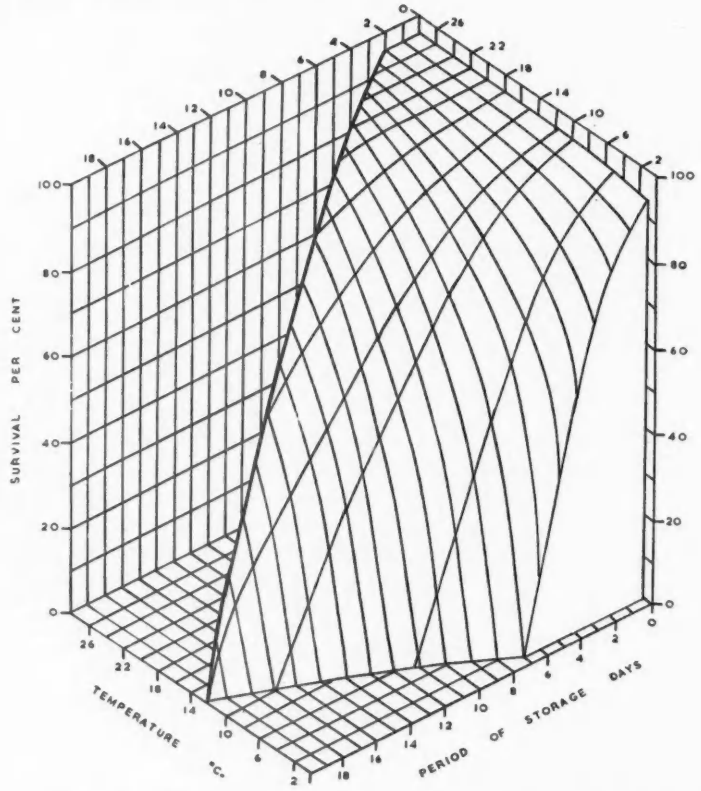

FIG. 3. *Isometric projection of curved surface representing the survival of puparia exposed to low temperatures.*

The Period of Highest Mortality in Stored Puparia

When puparia are subjected to cold storage only a certain percentage will survive to emerge as adults after being returned to a normal temperature of incubation. The percentage survival depends upon the intensity of storage. It was assumed at first that cold storage had some detrimental effect that resulted in mortality during the storage period. Dissection of the unemerged puparia, revealed, however, that death occurred when these were nearly mature, whereas, at the beginning of storage, the puparia were in an early stage of development. These observations suggested that mortality did not occur during storage but after the puparia had been returned to incubation.

A series of samples of 200 puparia from a single population were, therefore, stored for 10 days at 6.0° C.; a temperature well below the threshold of development. During the storage period and subsequent incubation at 26.7° C., samples were dissected to determine the extent of mortality and development. The results are shown in Table IV. Three arbitrary age classes were set up as follows; each of which is included in the table.

A—Age, 24 to 72 hr. of development.
Compound eyes, if formed, still white in color.

B—Age, 72 to 96 hr.
Compound eyes amber to light brown in color.

C—Age, 96 to 140 hr.
Compound eyes rusty brown to red.
Body and appendages well-formed, fuscous.

TABLE IV

CONDITION OF PUPARIA DURING STORAGE FOR 10 DAYS AT 6.0° C. AND SUBSEQUENT INCUBATION AT 26.7° C.

Time sample dissected	Alive, %			Dead, %			Total No. (100%)
	A	*B*	*C*	*A*	*B*	*C*	
Before storage	80	18	0	2	—	—	200
Immediately after storage	82	18	0	0	—	—	200
After: 24 hr. incubation	30	60	5	5	—	—	200
48	6	38	52	4	—	—	200
72	Emergence begun, no samples						
Emergence complete	0	0	0	5 (45% emergence)	0	50	200

About 5% of the population died at an age of less than 72 hr. This portion is probably identical with the 5 to 10% that dies even when no storage treatment is applied. The remainder of the mortality occurred within 40 hr. of emergence when the development was well advanced and the pupae were much like adults in appearance.

Similar results were obtained for storage temperatures of 1.0° C. and 9.4° C.

At temperatures above 1.0° C., therefore, mortality does not normally occur during storage but during subsequent incubation, and at a stage in development when the puparia are nearly mature.

The mechanism by which low temperatures exert a lethal effect upon puparia is not well understood. It can hardly be a simple one, since the

lethal effects are not manifested while puparia are in storage (at least, for periods of storage used in the present experiments) but only after metabolism is resumed and they have reached a certain stage of development. It appears likely that either the relative rates of various metabolic processes are changed to cause disorganization of the normal sequences of ontogeny at emergence or that some system or its precursor, important to the mature pupa, has been affected.

The Influence of Incubation on Survival of Puparia After Cold Storage

The Incubation Container

When puparia, following storage treatment, were incubated in a standard 10 cm. bacteriological Petri dish complete with cover, it was observed that the proportion that produced adults was higher than expected. It was also observed that puparia, incubated in 1 × 6 in. open shell vials, had a higher adult emergence than those incubated in open Petri dishes. Accordingly, several large series of samples of 500 puparia were withdrawn from a population, stored in an identical manner, and incubated in these three different ways. Adult emergence in covered Petri dishes or in shell vials was significantly higher than in open Petri dishes. There was no statistically significant difference between adult emergence in closed Petri dishes or shell vials.

Since incubation in a closed Petri dish was more convenient than in an open dish surrounded by a large screen cage to confine the emerging adults, this method of incubation was frequently used. Incubation in shell vial was used where periodic counts of emergence were required. The open end of the vial was projected through the wooden end of a small screen cage (6 in. × 4 in. × 2 in.) and held slightly inclined during incubation. Soon after emergence, adults entered the cage, which could be quickly changed when necessary, the hole being stopped with a cork. The cages were autoclaved to kill the emerged adults, which were sexed and counted.

During the course of the studies the number of living flies emerging from a large number of puparia was determined. Incubation was carried on in closed Petri dishes or in 6 in. shell vials. The survival curves derived from the experimental results are shown in Fig. 4. Survival is used here to designate the calculated proportion of living puparia as determined from the number of adult flies produced. It must be borne in mind, however, as in former experiments, that those that died during incubation subsequent to cold storage were included in the total number that succumbed. Thus, the puparia that succumbed between the stage in their development when they were removed from storage and emergence of the adults are not considered as having survived.

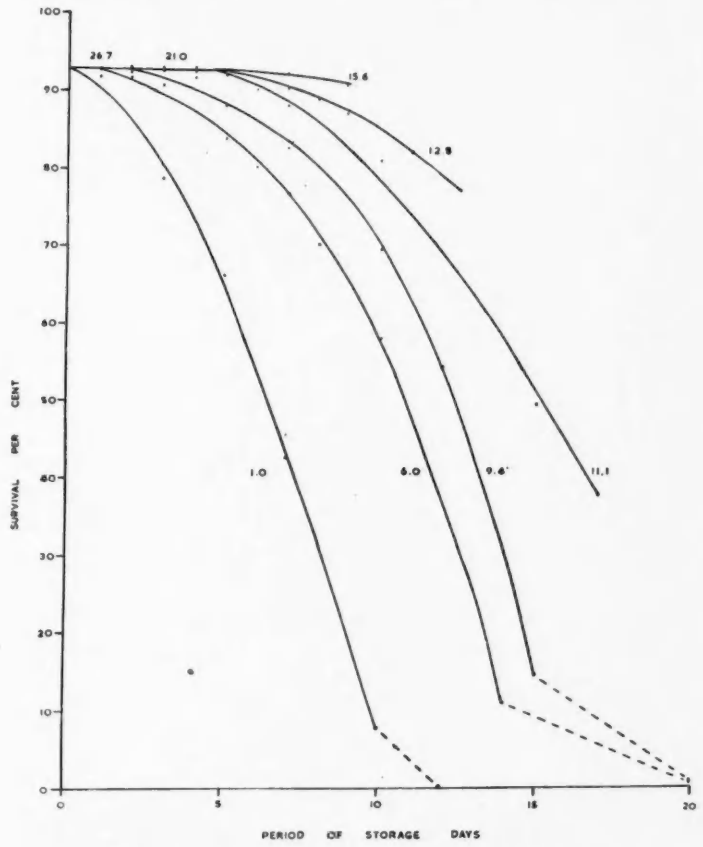

FIG. 4. *Survival of puparia exposed to low temperatures.*

By the use of multiple regression methods, similar to those used for incubation in open dishes, the equations of the survival curves, obtained by plotting survival (Y) against period of storage in days (X), were obtained. These equations are as follows:

Temperature	Equation	R
1.0° C.	$Y = 91.65 - 0.9309X - 1.07706X^2 + 0.03332X^3$	0.9993
6.0° C.	$Y = 94.03 - 1.4397X + 0.01057X^2 - 0.02356X^3$	0.9766
9.4° C.	$Y = 97.52 - 3.0369X + 0.40271X^2 - 0.03780X^3$	0.9863
11.1° C.	$Y = 89.74 + 2.2009X - 0.37626X^2 + 0.00383X^3$	0.9988
12.8° C.	$Y = 92.26 + 0.3059X - 0.04370X^2 - 0.00625X^3$	0.9998
15.6° C.	$Y = 92.60 - 0.0170X + 0.01959X^2 - 0.00416X^3$	0.9999

Since there was no significant difference between emergence from puparia stored at 21.0° C. and those incubated immediately at 26.7° C., survival at temperatures within this range is independent of the temperature and the equation for survival becomes $Y = a = 92.6$.

Using these equations estimated values of survival were calculated for different intensities of storage. The estimated survival figures were used to plot a series of curves showing the variation of survival (S) with temperature (T) for various periods of storage. The equations of these curves are given as follows:

Period of storage	Equation
1 day	$S = 89.70 + 3.730 \log T$
2 days	$S = 85.80 + 6.680 \log T$
3	$S = 80.00 + 11.820 \log T$
4	$S = 72.80 + 17.400 \log T$
5	$S = 64.19 + 25.317 \log T$
6	$S = 54.76 + 32.579 \log T$
7	$S = 43.95 + 41.158 \log T$
8	$S = 32.18 + 50.154 \log T$
9	$S = 19.71 + 59.237 \log T$
10	$S = -7.98 + 82.341 \log T$
12	$S = -63.03 + 125.970 \log T$
General	$S = a + b \log T$

Since the constants a and b varied progressively with the storage period, regressions between a and b and the period of storage (X) were calculated.

$$a = 98.65 - 8.32855X + 0.859359X^2 - 0.107229X^3 \quad R = 0.9991$$
$$b = -4.3641 + 7.38835X - 0.787839X^2 + 0.089650X^3 \quad R = 0.9987$$

Substituting the equations for a and b in the general survival temperature equation $S = a + b \log T$, an equation for survival in terms of both temperature and period of storage was obtained:

$$S = 98.65 - 8.32855X + 0.859359X^2 - 0.107229X^3 + \log T$$
$$(-4.3641 + 7.38835X - 0.787839X^2 + 0.089650X^3)$$

In Table V, survival calculated from the above equation is shown for the experimental temperatures for periods up to 15 days. There was good agreement between the calculated and observed values of survival. An isometric projection of the survival surface is given in Fig. 5.

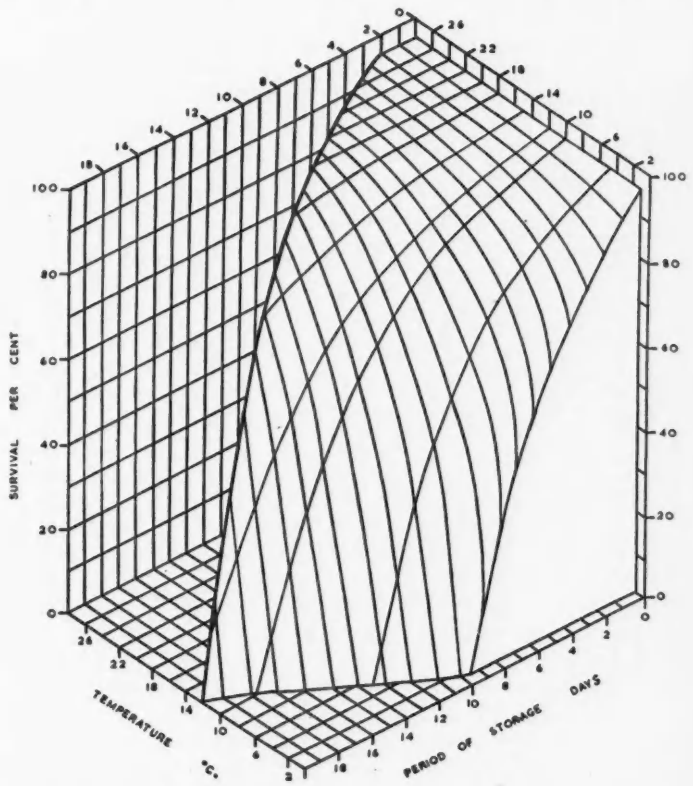

FIG. 5. *Isometric projection of surfaces representing the survival of flies from puparia exposed to low temperatures.*

From a consideration of these results it is clear that when puparia subjected to different intensities of cold storage are incubated in closed Petri dishes or small shell vials survival of the puparia, although differing somewhat from the survival of the puparia as shown in the previous set of experiments, remains as in the former case dependent upon the temperature of storage and duration of the storage period.

A comparison of the two sets of results (Tables III and V) shows that survival was higher when incubation was carried on in closed Petri dishes or in 6-in. shell vials rather than in open dishes with free access to the air. At temperatures from 21.0° to 26.7° C., the type of incubation container did not influence survival. As the intensity of storage was increased, by lowering the temperature or prolonging the exposure, the difference in survival between the two types of incubation increased to a maximum. With further increases in storage intensity the difference in survival became less marked.

TABLE V

CALCULATED VALUES FOR SURVIVAL OF PUPARIA INCUBATED IN CLOSED PETRI DISHES OR 6-IN. SHELL VIALS

Period of storage (days)	Temperature of storage (° C.)					
	1.0	6.0	9.4	11.1	12.8	15.6
1	91.1	92.9	93.3	93.5	93.6	93.8
2	84.6	90.6	92.1	92.7	93.2	93.9
3	78.5	88.7	91.3	92.2	93.0	94.1
4	72.2	86.5	90.1	91.4	92.5	94.1
5	65.1	83.8	88.5	90.3	91.8	93.8
6	56.5	80.6	86.6	88.8	90.8	93.4
7	45.7	76.4	84.1	87.0	89.4	92.8
8	32.1	71.2	81.0	84.6	87.7	92.0
9	15.1	64.7	77.1	81.7	85.6	91.1
10	—	56.6	72.3	78.1	83.1	
11		46.8	66.5	73.8	80.0	
12		35.0	59.6	68.6		
13		21.0	51.3	62.6		
14		4.6	41.8	55.5		
15		—	30.7	47.4		

It appeared that the superiority of the closed method over the open was related to the gaseous conditions of the atmosphere surrounding the puparia in incubation, probably being a function of differences between the concentration of oxygen, carbon dioxide, or other gaseous metabolites. Since no means of gas analysis were available, this hypothesis was tested partially by experimentation with a variety of incubation containers and population densities, in an attempt to secure variation in the gaseous content of the containers. Population densities between 500 and 10,000 were used. Incubation containers used varied from 6 in. × 2½ in. metal cylinders with screen sides and cardboard cylinders to glass flasks of assorted sizes, either open or partially or completely stoppered. The results are shown in Table VI.

TABLE VI

EMERGENCE OF FLIES FROM PUPARIA STORED AT LOW TEMPERATURES AND INCUBATED IN DIFFERENT CONTAINERS AND AT VARIOUS POPULATION DENSITIES. (THE PUPARIA WERE FROM NORMAL POPULATIONS STORED 10 DAYS AT 9.4° C.)

Container	Number of puparia	Emergence of adults, %
Petri dish open	500	51.6 (from Table V)
Petri dish closed	500	72.3 (from Table IX)
Screen and metal can	500 to 10,000	50.5
Metal cylinder, open mouth	5000	55.0
500 cc. Erlenmeyer flask open	500	64.4
	1000	60.7
	2000	55.7
	3000	21.7
	5000	4.7
500 cc. Erlenmeyer flask stoppered	1000	0

In all cases where comparatively free exchange of air was possible, survival was equivalent to that in open Petri dishes. Where diffusion was somewhat more limited, as in cardboard cylinders and metal cylinders with open mouths or open mouth flasks, survival increased though never equalled that of closed Petri dishes. Where diffusion was strictly limited, as in a closed or semi-closed container with a large population, survival was very low.

The Incubation Temperature

The standard temperature used for all incubation was 26.7° C. It has been shown by the storage experiments that incubation between 21.0° C. and 28° C. had no significant effects on puparial survival and observations indicated that extremely high temperatures of incubation invariably decreased survival of the puparia as shown by the reduction in the proportion producing adults. Where populations of 5000 to 10,000 puparia were incubated in thermos bottles, the heat of metabolism accumulated sufficiently to raise the temperature of the puparial mass to 40° C. or more. Under such conditions survival was nil.

Humidity During Incubation

When incubation was performed in closed Petri dishes and shell vials, water of transpiration and the meconial fluid of the emerging adults created dampness among the incubating puparia. To test the possibility that this high humidity was the most important factor in the greater survival in these incubation containers, puparia were stored for 10 days at 9.4° C. and incubated in small wooden boxes lined with blotting paper having sliding glass lids. In one series the blotters were kept dry and the relative humidity was 50%. In the other series the blotters were dampened and the puparia mixed with moist sawdust. The relative humidity was 100% and some free surface moisture was present. Survival was slightly better under conditions of high humidity but not sufficiently so as to explain the superiority of closed Petri dish incubation. Survival in incubation at 100% relative humidity was 65%, at 50% relative humidity it was 60%, and in Petri dishes 75%.

Storage Conditions Influencing Survival

Puparia were much less susceptible to changes in physical conditions during storage (except for temperature) than during the incubation period. Thus, the storage container and the gaseous concentration of the microhabitat could be varied within wide limits without affecting survival.

Puparia were stored at low temperatures in open or closed Petri dishes and shell vials in populations of 500, in metal and screen cans in populations as high as 15,000, and in large Petri dishes or tin boxes in numbers up to 50,000 without appreciable influence on survival, so long as sufficient diffusion occurred to maintain gaseous products below a lethal level and supply oxygen. When populations of 5000 to 10,000 were stored in tightly stoppered flasks, they all died, death occurring during the storage period, not in the incubation period.

Humidity was the most important factor during storage. Wide variations from 40 to 80% relative humidity produced no effect on survival. When, however, puparia were stored in moist sawdust, where the relative humidity was 100% and excess superficial moisture was present, mortality was greatly increased and survival was reduced to half the normal value.

The Influence of Larval Rearing on Survival of Puparia During Cold Storage

During the course of the experiments it was found that even when the physical factors of storage and subsequent incubation were constant the results in the case of certain populations of puparia varied considerably from those expected from any given storage intensity. Consequently, some biological factors known to be of importance in rearing were investigated with a view to determining their influence upon survival of puparia in storage.

Although rearing experiments had shown that increased age of the females reduced egg hatchability and survival of the larvae (*vide* first paper in series), in the present study tests on the progeny of three generations of females over a period of 26 days, no correlation was obtained between the age of the mother and resistance of her puparial progeny to cold storage. Similarly, the sex ratio of adults emerging from stored puparia was never significantly different from the sex ratio of unstored puparia. In storage experiments on 10,820 puparia at 6.0° C. for 15 days the sex ratio of the emergents was 50.7% females. Mortality appeared to be alike for both sexes. There was also no correlation between the size of the puparia and survival after cold storage. Small (0.006 gm.) and large (0.025 gm.) puparia produced the same number of adults following exposures to low temperatures.

Small changes in the constituents of the rearing medium such as those due to errors in measurement had no significant effect on survival of puparia after cold storage. If all or part of the alfalfa were replaced by an equal amount of sawdust, however, flies could be reared but the puparia showed a decreased ability to produce adults either when incubated directly or after cold storage. This was shown in a series of tests on 12 populations of flies reared on sawdust medium each of which was paired with a population reared on standard medium as a control. Puparia were subjected to three days' storage at 15.6° C. The differences obtained are shown in Table VII. Statistically they are highly significant.

TABLE VII

DIFFERENCES IN SURVIVAL OF PUPARIA REARED ON STANDARD AND SAWDUST-CONTAINING MEDIA, %

Mean emergence from standard medium	93.0
Mean emergence from sawdust medium	81.6
Mean difference	11.4
Number of paired observations	12.0
Value of P	<0.01

At greater intensities of cold storage the susceptibility of sawdust-reared puparia was even more marked. Sawdust-reared puparia stored for 10 days at 6.0° C. gave 10.0% emergence as compared to normal emergence of 56.6% and at 9.4° C., emergence was 28.0% with 72.3% emergence from the controls. It is clear that larvae reared on substandard media produce puparia that are more readily killed by low temperatures.

Under standard conditions of rearing, one battery jar of rearing medium was seeded with 2500 eggs or one small temperature-controlled box with 10,000 eggs. At this density about 72% of the eggs produced puparia. At greater densities (up to 5000 eggs per jar unit of rearing medium) the yield was decreased and the size of the puparia diminished. To determine whether rearing under these conditions altered the susceptibility of puparia to low temperatures during storage, a series of experiments was carried out in which puparia were produced at densities of 2500, 3000, and 3500 eggs per jar unit. These were subjected to five days' storage at 6.0° C. and incubated in closed Petri dishes. Emergence was not significantly different for the three rearing densities. Further trials indicated that, at a density of 5000 eggs per jar unit, adult emergence after storage was still not significantly different from normal.

The influence of larval-rearing temperatures on the resistance of puparia to cold is difficult to determine, since it is almost impossible to rear flies under accurately controlled temperature conditions. Daily changes in the temperature of the medium brought about largely by differences in the rate of fermentation, lateral and vertical gradients, and changes in the temperature preferences of the larvae made it impossible to make correlations between the resistance to storage and the temperature of rearing within the normal temperature range. Under abnormal conditions, however, where the mass of the rearing medium during the larval feeding period remained at 46° C. or more for some time, adult emergence from puparia subjected to low temperatures was noticeably decreased.

In one series of five observations emergence from puparia reared at the normal temperature range was 69.9% following storage at 9.4° C. for 10 days, while emergence from puparia reared where the medium was maintained at 45° to 46° C. for most of the feeding period was 59.8%. The two populations were of the same age when stored. At higher rearing temperatures and greater storage intensities the difference was more marked. Even when puparia were incubated for adult emergence at normal temperatures without storage treatment, a small but significant difference in emergence occurred, when rearing took place at abnormally high temperatures.

In all the experiments it was clear that high temperatures during larval rearing increased the susceptibility of the puparia to death at low temperatures. Although in some of the experiments it was not possible to attribute

the results solely to the direct effects of rearing temperatures, since the relative age of the puparia would be altered, it would seem that rearing the larvae at low temperatures provided the puparia with greater resistance to the detrimental effects of low temperatures at a later period in life. Whether or not the lower survival of puparia from larvae reared at high temperatures was due to the lack of acclimation, or the possibilities of reducing mortality by slowly lowering the storage temperature, was not included in these studies.

The Relationship Between Low Temperature and Rate of Puparial Development

Housefly puparia stored at temperatures above 11.1° C. produce adults, but below 9.4° C., if exposed for periods at least longer than 24 hr., none emerge. Emergence records indicated that puparia stored at 11.1° C. or higher undergo some development, but that no development occurs during storage at 9.4° C. or at lower temperatures. The threshold of development for puparia, therefore, must be between 9.4° C. and 11.1° C.

In order to define the threshold more precisely and obtain information upon the rate of development above the threshold temperature, 40 duplicate samples of 500 puparia were withdrawn from a single population, placed in shell vials, and stored at various temperatures for periods from 3 to 10 days. They were then returned to an incubation temperature of 26.7° C., the time of return being referred to as zero. Using small screen cages the emergents were counted every eight hours and the sexes recorded. For each sample the mean time for emergence for both males and females was then calculated in hours at 26.7° C. This time was generally two hours shorter for males than for females. The mean of the two times was taken as correctly representing a sample containing equal numbers of males and females. Where two duplicate samples did not differ by more than four hours, the mean of the two was accepted as the best measure of time for emergence for each specific treatment.

After storage at temperatures above 12.7° C., the time for emergence was less than for puparia incubated immediately at 26.7° C., the difference in time representing the amount of development that occurred during the storage period. Time for emergence or amount of development, in all cases, was measured in hours at 26.7° C. Hours of development during storage divided by the days in storage gave the hours of development occurring in 24 hr. at the storage temperature. This method was used to indicate the velocity of development at the different temperatures, the values of which are shown in Table VIII.

Thus it can be seen that the speed of development increases as the temperature increases at the rate shown in Columns 1, 2, and 3. Over this range of temperatures the regression equation was $D = 0 = -4.46 + 0.04885T + 0.03801T^2 + 0.00001T^3$. $R = 0.9929$.

TABLE VIII

AMOUNT OF DEVELOPMENT (MEASURED IN HOURS AT 26.7° C.) OCCURRING DURING 24 HR. AT DIFFERENT STORAGE TEMPERATURES

	Storage temperatures, ° C.								
	26.7	25.0	21.0	19.4	17.0	14.0	12.7	10.0	9.4
Mean amount of development in hours in 40 lots of 500 puparia	24.0 24.0 24.0 24.0 24.0	17.5 20.7 22.2	14.5 15.2 14.1 14.2 14.5 15.0 13.9	10.7 9.5 9.9	7.4 7.6 6.3 7.3 6.6 7.8	2.7 1.9 3.4 3.2 3.3 3.9	2.4 1.9 2.1 2.8 3.2 2.6	0.45 0.00	0.0 0.0
1	24.1	20.7	13.4	10.9	7.4	3.7	2.3	−0.2	−0.6
2	23.8	20.9	14.0	11.2	7.0				
3						3.1	2.4	0.5	0.0

Note.—1. Calculated from $D = 4.46 + 0.04885T + 0.03801T^2 + 0.00001T^3$
2. Calculated from $D = -22.31 + 1.7265T$
3. Calculated from $D = -17.24 + 17.7544 \log T$
Where D *= hours of development.* T *= temperature ° C.*

When the equation is represented as a curved line as in Fig. 6, it is seen that the calculated value of D becomes zero when T is 10.2° C. Thus development ceases at a temperature of 10.2° C. and may be considered as the "threshold of development" of Peairs (14), Shelford (19), and others.

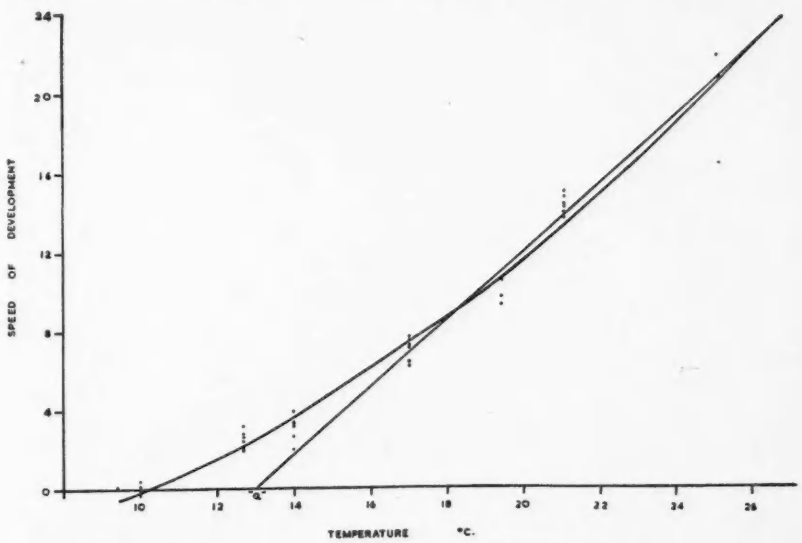

FIG. 6. *The rate of puparial development at temperatures from 0° to 26° C. The units of speed of development is shown in hours.*

Above 17° C. the relationship between speed and temperature may be expressed equally as well by a straight line, the equation of which is $D = -22.31 + 1.7265T$. The equation is represented graphically by the straight line shown in Fig. 6. If the straight line is extrapolated, D becomes zero when T equals 12.9° C., the temperature referred to by Wigglesworth (22) as "zero development" or the "*a*" point of Shelford (19). By calculation from the linear equation the thermal constant for fly puparia as determined by Peairs' method (14) of *thermal summation* was found to be 80.5 degree-days.

From the observations given above it may be concluded that for housefly puparia the relationship of rate of development to temperature is best expressed by considering velocity as a linear function of temperature above 17° C. and, below 17° C. as a linear function of the logarithm of the temperature. In the present study the upper limits of the biokinetic range were not investigated so that the beginning of departure from linearity, which commonly occurs at high temperatures, is not known. From the time of development calculated from data given by Feldman-Muhsam (8) on the development of housefly puparia at 29° and 34° C., it would appear that the relationship of speed of development and temperature is linear up to at least 34° C.

The Effects of Puparial Storage on the Adults

The storage of puparia at low temperatures not only caused a reduction in adult emergence but also had noticeable effects upon both adult life and the survival of their progeny. This was evident in varying intensities at different storage temperatures.

Puparia from 14 populations were subjected to 10 days' storage at 6.0° C., and then incubated at 26.7° C. Records were kept on oviposition and mortality of the emerging adults and on egg hatching and survival of filial larvae under standard rearing conditions. The results obtained compared with those from the normal breeding populations (p. 29) and with their standard deviations are given in Table IX.

TABLE IX

COMPARISON OF FLIES FROM NONTREATED PUPARIA AND ADULTS FROM PUPARIA STORED AT 6.0° C.

	Adults from nontreated puparia	Adults from puparia stored 10 days at 6.0° C.
Eggs per female per day (first 10 days of laying)	30 ± 10	5.7 ± 3.1
Percentage of population dead after 10 days of laying	50 ± 10	87.9 ± 7.1
Percentage of eggs that hatched	80 ± 4	60.0 ± 3.0
Percentage of survival of larvae	90 ± 8	52.9 ± 16.0
Percentage survival from eggs to puparia	70 ± 9	34.9 ± 15.1

In the table it can be seen that adult populations, emerging from puparia stored 10 days at 6.0° C., were distinctly abnormal. Adult mortality during the first 10 days of oviposition was high, egg deposition was low, and the egg hatch was low. Larvae, emerging from eggs, did not survive well in rearing while the over-all yield of puparia was only about half that from untreated flies.

At higher storage temperatures the results were somewhat similar and a more detailed study of the effects of puparial storage was, therefore, carried out at 9.4° C. Puparia from a single population were stored at 9.4° C. for different periods from 0 to 10 days. The emergent adults were placed in standard screen cages at population densities of 500 per cage and continuous records obtained similar to those in the previous experiment. The results obtained are given in Table X. The regression equations obtained from the 12 observations are given at the end of the table. In every case the probability levels were well below 0.01 and the regression coefficients were, therefore, highly significant.

TABLE X

EFFECTS OF PUPAL STORAGE AT 9.4° C. ON ADULT LIFE

Cage number	Days in storage	Eggs per female		Percentage of total eggs laid in first 10 days	Hatch, %	Percentage of flies dead in first 10 days			Length of adult life (days)	
		Total	Per day for first 10 days			♂	♀	Population with equal sexes	♂	♀
1	0	526	32.0	60.9	77.0	80.4	31.1	55.7		
2	0	424	24.8	58.5	71.2	91.8	40.3	66.0		
Mean	0	475	28.4	59.7	74.1	86.1	35.7	60.9	10.2	18.2
3	2	356	24.0	67.2	69.2	84.2	43.0	63.6		
4	2	385	25.5	66.2	70.2	86.1	44.0	65.0		
Mean	2	370	24.7	66.7	69.7	85.1	43.5	64.8	10.1	15.6
5	4	321	25.2	78.4	57.9	90.9	47.7	69.3		
6	4	246	17.4	70.9	65.0	94.8	50.6	72.7		
Mean	4	284	21.3	74.6	61.4	92.8	49.1	71.0	9.3	14.6
7	6	321	27.3	85.2	54.3	93.1	65.7	79.4		
8	6	296	24.8	83.9	51.6	93.7	60.3	77.0		
Mean	6	308	26.0	84.5	52.9	93.4	63.0	78.2	8.2	12.1
9	8	179	14.7	81.8	46.4	93.3	58.7	76.0		
10	8	231	20.5	88.7	45.5	94.6	66.5	80.5		
Mean	8	205	17.6	85.7	45.9	94.0	62.6	78.3	8.6	12.3
11	10	127	12.2	96.1	36.8	96.4	82.4	89.4		
12	10	116	11.2	96.1	34.5	96.6	87.2	91.9		
Mean	10	122	11.7	96.1	35.7	96.5	84.8	90.6	7.1	9.3
Regression equations		$Y=454-32X$	$Y=28.8-1.43X$	$Y=60.1+3.54X$	$Y=76.0-3.89X$	$Y=85.6+1.13X$	$Y=33.9+4.52X$	$Y=59.7+2.83X$	$Y=10.4-0.30X$	$Y=17.7-0.81X$

Similar experiments were carried out at 15.6° C. At this temperature male emergents from stored puparia were relatively little affected by storage up to at least eight days but females laid fewer eggs and appeared to have a slightly shorter adult life than normal stock. This is shown in Table XI. Although regression equations were calculated for the number of eggs deposited per female ($Y = 683.4 - 33.6X$) and the length of adult female life ($Y = 18.9 - 0.68X$) only the coefficient for egg deposition was significant.

TABLE XI

EFFECTS OF PUPAL STORAGE AT 15.6° C. ON ADULT LIFE

Cage number	Days in storage	Total eggs per female	Length of adult female life (days)[a]
1	0	797	21.9
2	0	644	16.4
Mean	0	720	19.2
3	2	642	20.1
4	2	522	15.4
Mean	2	582	17.8
5	4	506	15.8
6	4	545	14.9
Mean	4	526	15.4
7	8	430	13.5
8	8	440	14.1
Mean	8	435	13.8

[a] *The records of male longevity are not presented since they were similar to those obtained in the case of nontreated males (vide page 20, Part I of this series).*

From these experiments it is clear that subjecting fly puparia to low temperatures not only caused mortality during the pupal stage but affected the adults that survived the treatment and emerged. Thus, surviving adults had a shorter life and deposited fewer eggs after having passed through a period of cold during the pupal stage. Moreover, the survival of their progeny was noticeably reduced, possibly by a decreased vitality of the larvae. It would appear that in this species the reproductive system and particularly that of the female is especially sensitive to harmful effects of cold temperatures.

Discussion

For many insects the velocity of development is a linear function of the *effective* temperature over a certain portion of the "zone of effective temperatures" (Chapman (7)). The velocity-temperature curve departs from linearity, however, as the minimum and maximum effective temperatures are approached, as shown by Krogh (9). For housefly puparia, between 17° and 34° C., the rate of development is clearly a linear function of temperature but below 17° C. the rate varies with the logarithm of the temperature.

In the present study the effect of low temperatures on the survival of housefly puparia show that between temperatures of 1° C. and 20° C. the mortality of puparia is dependent upon both time and temperature. Equations have been calculated that show that, when temperature remains constant, the mortality varies with time according to equations of the form; $S = a + bX + cX^2 + dX^3$ and that, when time remains constant, the mortality varies with the temperature according to equations of the form $S = a + b \log T$. It has also been shown that the effects of time and temperature can be combined in a single survival equation and that this equation may be represented graphically by a curved surface.

Below 1° C. the effect of temperature on puparia was not investigated. That puparia are very susceptible to temperatures below 0° C. was shown for a small number of samples by Feldman-Muhsam (8). All puparia died after 12 hr. at −3° C., 3 hr. at −7° C., and 1 hr. at −11° C.

Between 20° C. and 28° C., survival of puparia is constant and may be represented by the equation $S = a$, where S is survival and a is a constant having the value 92.6 + 2.4 for the Powell strain, used in the present study.

Above 28° C. the effect of temperature upon puparia was not investigated. Feldman-Muhsam (8), who incubated puparia at 34° C. does not indicate that survival was affected. During the course of the present study it was observed that, when incubation took place in thermos flasks, heat of metabolism was sufficient to raise the temperature to 40° C. or higher. When this occurred, survival was very low.

A number of theories have been presented to explain the killing action of low temperatures. These have been reviewed by Bělehrádek (3). In the case of insects that are very resistant to cold, death is apparently the result of ice-formation in the tissues, although some insects can apparently withstand even complete freezing. Resistance to cold, in these insects, is apparently correlated with the prevention of supercooling. In insects that are not very resistant to cold death occurs before freezing of the tissues, provided the time factor is sufficiently long. The mechanism causing death, in this case, must be widely different from that operative during freezing. Bělehrádek points out that a number of changes occur in cells when they are chilled and reviews the literature in this connection. Chief among these probably are an increase in viscosity of the protoplasm and abnormalities of mitotic division.

Early theories explaining the mechanism causing death by temperatures above freezing were based on the hypothesis that there was a disproportion between the velocities of several vital functions. Many workers believed that the effect was a result of the accumulation of toxic products that were eliminated at higher temperatures. Bělehrádek (2) proposed the hypothesis that an increase of protoplasmic viscosity was responsible for the action of cold. Such an increase in viscosity would hinder molecular movement and affect the rate of biological processes in the organism.

From the results obtained in the present investigations any attempts to explain the mortality of housefly puparia subjected to periods of cold storage must take into account the following experimental observations:

(1) There are variations in resistance to storage between individuals of a population.

(2) Mortality increases as the storage temperature decreases and as the duration of storage increases.

(3) Puparia are not killed by exposure to storage for considerable periods but resume development on being replaced in a favorable temperature, and die only when a definite stage of development has been reached.

(4) The gaseous concentration of the atmosphere during incubation has an influence on mortality following a given storage period, but is a relatively unimportant factor during the storage period.

(5) The age of the puparia, when placed in storage, has a marked effect upon survival.

(6) Storage at temperatures above the threshold of development results in some mortality.

(7) Puparia produced under conditions of high temperature are more susceptible to cold storage.

(8) The individuals surviving a period of cold show evidence of disturbed physiology in the adult stage as evidenced by their shorter life and lowered fecundity.

(9) The reproductive system appears to be affected to a greater extent than other systems, since a reduction of oviposition occurs after storage temperatures that have little effect on longevity. Moreover, the progeny of stored puparia show decreased vitality, there being a reduction of egg hatch and survival of larvae in rearing.

It would appear that an explanation based on the effect of temperature upon the rates of biological processes would best fit these observations.

In studying the effect of temperature on the duration of development in *Drosophila*, Powsner (16) visualized development as the result of a complicated multidimensional network of physical and chemical processes, acted upon both by the genetic make-up and by the environment. He explained the departure from linearity at the extremes of the velocity–temperature curve as follows: "When many reactions proceed simultaneously and only interact at certain points, it is conceivable that all of them will maintain proportionate rates over a long temperature range. Toward both extremes of this range these reactions are likely to become more and more disorganized, so that development becomes abnormally prolonged at these temperatures and both ends of the normal van't Hoff rate curve depressed. . . ."

It would seem that development in the housefly may also be considered the result of a network of interacting processes, all more or less of equal importance. The application of low temperature to this system would probably affect the

rates of some processes more than others, so that the whole system would be disorganized. Beyond a critical point recovery from disorganization does not occur and the result is death.

This may be amplified by further reference to the experimental observations given above.

(1) Individual variations in resistance may be due, either to individual variation in the ability to recover from disorganization or to individual variation in the amount of disorganization brought about by a particular storage treatment. They may be caused by differences in genetic make-up, past environmental experience, or age. The experimental evidence suggests that the last two factors are most important.

(2) If the application of low temperatures causes a disproportion in the rates of ontogenetic development and leads to disorganization, it can be seen that the lower the temperature or the greater the duration of the storage treatment the greater will be the degree of disorganization. More individuals will have passed the critical period of recovery and mortality will therefore increase.

(3) The effect of disorganization of the developmental system need not be apparent immediately. It is more likely that the lethal effect should become manifested towards the end of the pupal period, when the final reactions are poorly synchronized. Since the end of the period is marked by a critical stage in the history of the animal, namely, the emergence of the adult, it is not surprising that death should occur chiefly at this point.

(4) It is more difficult to reconcile the hypothesis with the observation that the gaseous composition of the atmosphere during incubation has an effect on survival. Unpublished data from the Belleville laboratory demonstrate that puparial development in the houseflies can be retarded by placing the puparia in partial vacuum or a medium of high nitrogen concentration. It would seem that when puparia are incubated in shell vials or closed Petri dishes the composition of the atmosphere is such that retardation of some of the developmental processes occurs to partially reverse the temperature effects. During the storage period, when metabolism is low, wide variations in atmospheric composition would not have much influence on survival.

(5) At different periods during the development of pupae, changes in reactions and in the relative rates of reactions can be expected. Bell (4) has shown that the rate of oxygen consumption in pupae of *Galleria mellonella* varied with age, and was at a minimum when pupae were about one-third developed. Similar curves were obtained by Krogh (9) for carbon dioxide production by *Tenebrio molitor* pupae at different constant temperatures. It can be expected, therefore, that the application of low temperature at one period would cause a greater disproportion between rates of reactions than at another period and would consequently result in greater disorganization. In general, greater disorganization of the reactions just previous to emergence would be expected, if the factor causing the disorganization were applied very early in the pupal stage and were cumulative.

(6) Since the rate curve for development of housefly puparia begins to depart from linearity at about 17° C., it would be expected that any temperature below this would disproportionately affect the rates of the various reactions involved. Long storage at temperatures below 17° C. might therefore disturb the network of reactions and result in some mortality, even though development would progress and some emergence occur. It has been shown that some mortality does occur at 15.6° C. Although no experimental data are available between 15.6° and 20° C., it can be seen from the puparial survival shown in Tables V and VIII that little mortality occurs between 17° C. and 20° C. and none above 20° C. where the rate curve is linear.

(7) The temperature at which organisms are reared may influence their ability to withstand cold and it has been suggested that the essential factor is the solidification point of protoplasmic lipoids, which in turn depends on the temperature at which they are formed. It would seem that this is the explanation for the observation that housefly puparia reared at very high temperatures are more susceptible to cold. It may also be that in flies reared at high temperatures the rates of developmental processes are increased and that the processes with increased rates are more susceptible to the disorganizing action of low temperatures.

(8) and (9) If a partial disorganization occurs at the time of adult emergence, it is not surprising that some should be retained in the adult stage and that certain evidences of the disturbance should be apparent in adult life. Disturbed adult physiology in the housefly is shown by a shorter adult life and by impaired fecundity. The reproductive system, particularly that of the female, is apparently more readily disorganized by low temperatures than other parts of the body. Thus oviposition is low after puparial storage. The hatching of the oviposited eggs is also below normal. Whether this is due to decreased fertilization or to factors more closely associated with the female (such as the quantity of yolk) was not determined. At any rate both eggs and the emerging larvae show lowered vitality. By the time pupation has occurred the individuals showing evidence of disturbed physiology have been eliminated and adult emergence from these puparia is normal.

It would appear, therefore, that mortality of housefly puparia, subjected to cold storage, occurs as the result of a disorganization of the interacting network of processes making up development. The disorganization is brought about by a change in the relative rates of the processes of ontogeny at temperatures below 17° C. Its magnitude depends chiefly upon the temperature, the duration of storage and the time (age) when storage is applied. Disorganization in the interaction of developmental processes increases progressively throughout pupal life until, at the time of emergence, the end processes are completely out of synchronization and mortality occurs.

In temperate climates, where the breeding of houseflies is interrupted by winter, considerable interest has developed in the question of how flies pass the winter. The majority of investigators believe that overwintering occurs

in the adult stage. The results of the present study indicate that the overwintering of flies as puparia is most unlikely, even though the freezing point of puparia was determined by Salt (18) to be about −12° C., or even as low as −22° C. after desiccation. At temperatures below the threshold of development puparia fail to produce adults after 20 to 25 days, while at temperatures above the threshold emergence occurs of at least a portion of the population. When adults emerge from puparia exposed to a period of low temperature, oviposition, egg hatch, and survival of hatched larvae are all so low that the chance of a filial generation surviving is very poor.

Unpublished results from studies at the Belleville laboratory have indicated that both eggs and mature larvae have little resistance to cold. Adults, however, may be kept alive for considerable periods between 10° to 15° C., although at temperatures much lower they also succumb to cold. It is considered most likely, therefore, that in temperate zones, houseflies pass the winter by a combination of adult hibernation and semicontinuous breeding in favorable situations.

No evidence has been found during this study to suggest that a condition of true diapause occurs during any stage of housefly development.

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STUDIES ON THE HOUSEFLY (*MUSCA DOMESTICA* L.)

III. THE EFFECTS OF AGE, TEMPERATURE, AND LIGHT ON THE FEEDING OF ADULTS[1]

By G. E. Bucher,[2] J. W. MacB. Cameron,[3] and A. Wilkes[4]

Abstract

For a short time immediately after emergence adult houseflies do not consume food. Flies begin feeding at a temperature and age at which they first become active, although to some extent it is dependent on the relative proportions of the sexes, the presence or absence of light, and the accessibility of food. There appears to be no upper age limit at which flies cease to feed. At 27° C. flies died in a very short time if they were not supplied with an abundance of liquid food. All flies died when starved for 24 hr. at 27° C. after having once been fed.

Introduction

For many insect pests a knowledge of their feeding habits has been the only reliable clue to the formulation of satisfactory measures of control. This is true in the case of the housefly and to a certain extent for other insects with somewhat similar habits injurious to orchard and garden crops. Thus the period and rate of the feeding activities, particularly in connection with environmental conditions such as weather has not only an important bearing on the application of control measures but is of practical interest in connection with damage, since in many cases it is due to the quantity and manner in which food is taken by the insect.

Among the saprophagous insects the housefly is probably the best known and it might be assumed that its feeding behavior has been thoroughly studied. This, however, is not the case. Apart from the investigations of Hewitt (4), Graham-Smith (3), Baumberger (1), and others on nutritional requirements and structural makeup of the mouth parts, little has been done on the feeding habits of the adults.

During the present series of studies on the housefly, *Musca domestica* L., general observations indicated the possibility of obtaining more precise information on feeding. In the laboratory rearing of flies it was noted that for some time after emergence adults were inactive and not readily attracted to food. Experiments were, therefore, set up in order to determine the duration of the inactive period under different temperature conditions with a particular view to a determination of the lowest temperature range for feeding and the effects of light and darkness. The purpose of the present paper is to present the results obtained.

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Experimental Methods

For the experiments, adults of the Powell strain were used throughout. These were secured from puparia reared in the laboratory and incubated at 26.7° C. In view of the differences in length of life, fecundity, and time of emergence of adults occurring between different populations of flies reared in the laboratory (*vide* Part I of this series*) all puparia used in the present study were withdrawn at random from well-mixed populations. The flies were collected at hourly intervals, placed in small boxes $5\frac{1}{2} \times 8\frac{1}{2} \times 1\frac{1}{2}$ in., and supplied with food consisting of the standard milk solution to which had been added a small amount of black India ink. The ink appeared to have no ill effects on the flies. At the end of each experiment the flies were killed in cyanide bottles, sexed, and examined by dissection for signs of feeding. The flies that had fed could be readily detected by the black coloration of the ingested food in the alimentary canal.

Results

In any sample population of flies of the same age there are differences between individual members with regard to the onset of feeding. They do not all begin to feed at the same time. A few begin feeding soon after emergence while others go without food for some time. Thus, at any given time up to about two days after emergence, only a certain percentage of the population has taken food for the first time. The extent to which they feed appears to be dependent largely upon the sex ratio, presence of light, and the temperature of the environment.

Under normal rearing-room conditions a few flies begin to feed soon after emergence from their puparia. Most of them, however, do not begin to feed until about six hours later and it is usually 24 hr. before they have all taken food. At lower temperatures feeding begins much later in adult life. This is shown in Table I. In the table are given feeding records obtained from 23,320 flies tested in environmental temperatures of 27°, 21°, 15°, and 10° C. The flies used in each experiment were taken from populations having equal proportions of males and females.

It may be seen in the table that the percentage of flies that feed increases gradually with age. The increase in rate when plotted against age, as illustrated in Fig. 1 for 27° C., is distinctly sigmoidal in form. At lower temperatures the curves become flattened until at 10° C. they are almost straight lines. Although the data for 15° C. are incomplete, it appears that very few flies begin feeding until they are over eight hours old but by the end of the first day and a half of adult life they had all consumed at least one meal. At 10° C. feeding was very erratic. Some of the flies did not feed for two and one-half days at this temperature. At 6° C. and 0° C. flies did not feed at any age.

*Wilkes, A., Bucher, G. E., Cameron, J. W. MacB., and West, A. S. Can. J. Research, D, 26:8-25. 1948.

TABLE I

FEEDING OF FLIES AT DIFFERENT TEMPERATURES

Age (hours from emergence)	Flies feeding, %			
	27° C.	21° C.	15° C.	10° C.
1	1	0		
2	6	1		
3	18	5		
4	32	10		
5	43	17		
6	51	24		
7	58	31		
8	64	36		
9	69	41		
10	73	46	19	
12	80	55	26	
14	86	62	35	
16	91	69	43	
18	94	75	52	
20	96	80	61	
22	97	85	69	
24	98	89	76	31
26	98	93	82	
28	98	96	87	
30	99	98	91	
36	99	99	98	68
60				94

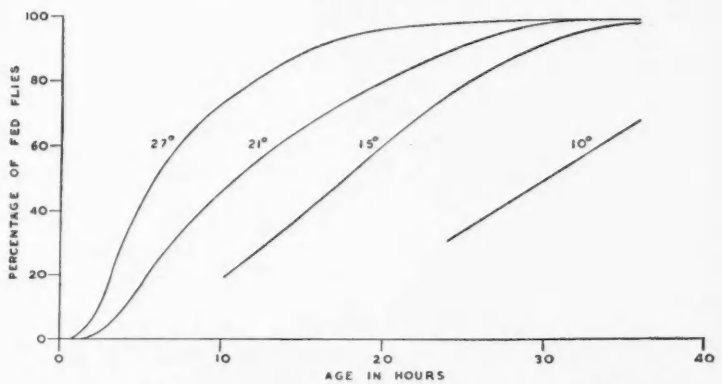

FIG. 1. *The feeding of flies at different environmental temperatures.*

At normal breeding room temperatures (27° C.) male flies begin to feed at a slightly earlier age than females. The difference in time between emergence and the first meal shown by the two sexes becomes greater at lower temperatures. This is shown in Table II. At 15° C. about one-quarter of the males had fed nine hours after emergence whereas less than 1/10th of the females had taken food. At 10° C. after one day, half the males had fed and only one quarter of the females.

TABLE II

DIFFERENCE IN FEEDING OF MALE AND FEMALE FLIES

Age (hours from emergence)	Males				Females			
	No. examined		No. feeding, %		No. examined		No. feeding, %	
	At 27° C.	At 21° C.	At 27° C.	At 21° C.	At 27° C.	At 21° C.	At 27° C.	At 21° C.
1	107	144	0.9	0.0	197	60	1.5	0.0
2	96	99	5.2	1.0	103	48	7.8	0.0
3	113	186	20.4	7.0	153	123	26.8	4.9
4	180	214	38.9	13.1	221	204	26.2	7.4
5	533	483	49.5	20.0	596	436	38.3	12.4
6	227	289	56.4	35.6	176	275	43.8	31.6
7	313	274	63.9	35.4	306	233	55.2	27.5
8	302	388	74.5	40.5	333	396	54.7	35.9
9	218	230	73.9	44.3	193	235	70.5	29.4
12	644	591	84.2	58.0	513	595	78.0	49.9
16		250		70.0		285		61.6
17	320		93.8		340		92.1	
18		372		76.6		382		72.5
19	101		99.0		69		98.6	
20	331	126	96.7	82.5	206	123	95.1	77.2
23	917	558	97.9	88.2	800	367	97.0	87.2
25		271		92.6		159		88.1
26		156		96.8		234		96.6
27	106	111	100.0	95.5	138	100	100.0	90.0
28		197		97.0		215		95.8
30	194		99.0		220		99.1	
36	703	577	99.1	98.9	666	543	97.1	98.0

The absence of light also appears to have a marked bearing on the feeding of houseflies. Experiments carried out in complete darkness and in normally illuminated laboratory rooms show (Table III) that a much smaller percentage of flies will feed in darkness than in the presence of light. Although a few flies, approximately 25%, consume some food in the absence of light, it is probably due to chance contact of the flies with the food dishes in the more heavily populated cages. The results of a series of feeding experiments at temperatures from 0° to 27° C. are shown in Table III.

TABLE III

FEEDING OF FLIES IN LIGHT AND TOTAL DARKNESS. THE FLIES WERE 36 HR. OLD AT BEGINNING OF THE EXPERIMENT

Temperature (°C.)	Percentage of flies feeding	
	In light	In darkness
27	99.4	27.4
21	99.3	20.6
15	97.9	25.2
10	67.8	7.7
6	0.0	0.0
0	0.0	0.0

Discussion

From the experiments reported here and from observations on numerous populations of flies handled during laboratory propagation it would appear that the feeding behavior of flies is closely associated with general adult activity rather than with anatomical or physiological changes, per se, in the adult. Thus, the temperature limits for feeding are apparently very near the limits for general activity and in this respect *M. domestica* appears to be similar in its feeding habits to grasshoppers. Observations on the feeding of grasshoppers by Parker (5) illustrate this point very clearly.

The temperature at which flies become active and at which they commence feeding is almost the same. At 27° C. about one hour is required after emergence for expansion of the wings and general hardening of the body. At this temperature the adults remain relatively quiescent for about the first four hours. Normal activity is reached in about 15 hr. At 21° C. the hardening process is somewhat slower. Adults remain inactive for from six to eight hours and full activity is reached in about a day. At 15° C. the process is still slower, flies never becoming active as at higher temperatures. Flies are very sluggish at 10° C. and show a much greater tendency to walk than fly. Little movement occurs at 6° C. Flies usually cling to the sides of the cage and only walk slowly when disturbed. At 0° C. there is very little coordinated movement, although the flies cling to the walls of the cage unless they are dislodged by jarring. In complete darkness they behave in much the same way, being very quiescent even at temperatures as high as 27° C.

As in the apple maggot fly, *Rhagoletis pomonella* Walsh (*vide* Caesar and Ross (2)), freshly emerged adults usually apply their mouth parts to the sides and floor of the cage and appear to be lapping up food. For the first hour or so, depending on the temperature, however, little or no food is taken into the body and none appears to enter the alimentary tract until the body and appendages have hardened. After this period the percentage of flies that feed is dependent upon the degree of activity of the population; the greater the activity, the greater the likelihood of the fly coming in contact with the food.

Throughout these studies there appeared to be no upper age limit at which the flies ceased to feed. Flies over 35 days old fed actively at 27° C. At the higher temperature the adults died in a short time if they were not almost constantly supplied with a very liquid diet. Almost all the flies died when starved for 24 hr. at 27° C. and after having once fed they were particularly susceptible to death from starvation.

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ACCESSORY TAILS IN FROG TADPOLES, THEIR EXPERIMENTAL PRODUCTION AND SIGNIFICANCE

I. FIVE CASES OF ACCESSORY TAILS IN TADPOLES OF *RANA CLAMITANS* AS A RESULT OF NATURAL INJURY[1]

By David J. McCallion[2]

Abstract

In a collection of several hundred *Rana clamitans* tadpoles, from a pond, five were found having accessory tails. These are described with a probable explanation of their occurrence.

Introduction

Many naturalists have observed and commented on the reproduction of lost tails by lizards. Pliny and Aristotle described some cases of many-tailed lizards. A variety of explanations of the production of accessory tails in lizards were offered by such early investigators as Cardane, Porta, Aldrovandi, Albert-le-Grand, and others. Among them was the belief that double tails were formed from imperfect double eggs. Cuvier discovered that the axial skeleton of a newly regenerated tail was largely cartilaginous, thus providing the clue to a sound explanation of the formation of more than one tail by regeneration. Gachet (6), Müller (9), Fraisse (5), and Tornier (11) produced accessory tails in lizards by experimental methods and contributed to the understanding of their formation. Gräper (7) offered the most satisfactory explanation of this phenomenon.

Accessory tails in urodeles seem to be less well-known. Terni (10) produced a ventral accessory tail in *Triton*. This tail was well-formed, lacking only a caudal artery. One specimen of *Triturus viridescens* having a ventral accessory tail was found and described by Dawson (4). He was able to produce the same result experimentally. Crummy (3) also reported a bifid tail in this species.

There seems to be no report of accessory tails resulting from natural injury in anuran tadpoles. Several investigators (Harrison (8), Barfurth (2), and Avel (1)) have produced accessory tails in these animals in the course of their experiments. Of particular interest in this discussion is the method used by Avel. He cut a hole through the side of the tail of *Rana temporaria* so that a short length of notochord was removed. A new tail grew out from this point, assumed the normal axis, and pushed the old tail aside.

Five Cases of Accessory Tails in Frog Tadpoles as a Result of Natural Injury

In the late fall several hundred tadpoles (*Rana clamitans*) were collected from a pond near McMaster University and taken to the laboratory for use in experiments. Among them were five tadpoles with accessory tails. It was

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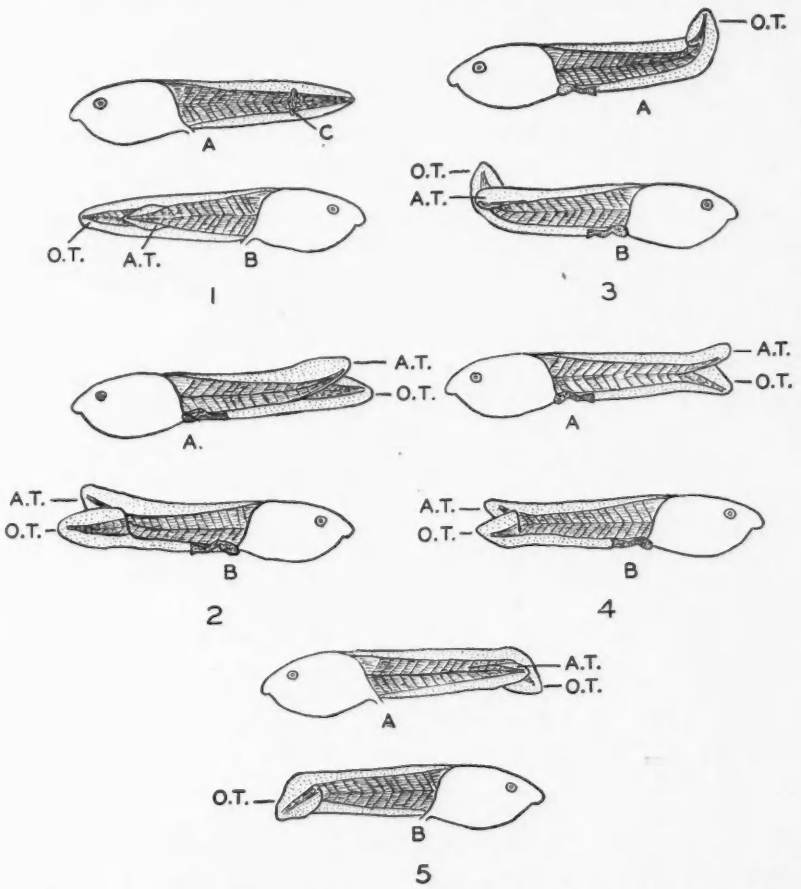
not possible to photograph them at the time, but the outlines of the tails were drawn and they are described below. These tails were sectioned transversely and examined histologically to determine the extent of regeneration of each of the injured tissues.

CASE I

On the left side of the tail, about one-third of the distance from the distal end, there was what appeared to be a small, irregular, cutaneous outgrowth (Fig. 1A). At about the same position on the right side of the tail there was a small, nicely-formed tail that had attained a length of about 6 mm. (Fig. 1B). It was directed posteriorly and lay almost in the normal axis. Since this



FIGS. 1 TO 5. *Free hand outline drawings to show the nature of accessory tails and old tail ends of tadpoles collected from a pond. Right and left sides of each are shown. Figure numbers correspond to case numbers used in the text. A = Left side; B = Right side; A.T. = Accessory tail; O.T. = Original tail; C. = Cutaneous growth.*

structure so closely resembles those described by Avel (1), it can be assumed that the tail of this tadpole had been pierced in the same way as in the animals that he described. Histological examination showed that the small growth on the left side of the tail was composed almost entirely of epidermis and mesenchyme. There were a few muscle fibers but no large vessels. In the tail-like structure on the right side there was a notochord that was a branch of the original notochord. Mesenchyme and muscle fibers were also present, but the nerve cord and caudal vessels were absent. The nerve cord of the old tail was continuous, suggesting that it had not been injured, and therefore, had had no opportunity for regeneration and could not appear in the accessory tail.

Cases II, III, and IV

Each of these animals had apparently sustained the same kind of injury, that is, a deep incision, downward from the dorsal edge of the tail, that severed the notochord. The resultant accessory tails appeared very much alike (Figs. 2, 3, and 4). In each case the tip of the tail seems to have been incompletely amputated. As a result, a new tail grew out by regeneration from the posteriorly directed surface of the wound while the old tail end remained attached to the stump by muscle and became consolidated with it at the site of injury. Thus, any possible regeneration from the anteriorly directed surface of the wound was mechanically prevented. In Case II the accessory tail was quite complete, except that the caudal vessels were irregular and there was no ventral fin fold. Nerve cord, notochord, and muscle were present. The old tail end, of course, had a normal appearance. The accessory tail and the old tail end in Case III were not very different from those in Case II. However, at the site of injury, where both regeneration and healing together of the tissues had taken place, growth was very irregular. At this point there were three notochords and four nerve cords. The true notochord and nerve cord of each tail could be easily identified. The others were simply irregular branches that were not present at more distal levels. In Case IV the old tail end and the newly regenerated tail were more completely united at the point of injury than in the other two cases. In both parts of the tail all of the normal tissues were present except that the nerve cord had not regenerated into the new tail. It was probably prevented from doing so by irregular growth of the notochord. There were two branches of the new notochord at the distal end of the accessory tail but at more proximal levels this appeared as a large mass. The nerve cord probably abutted on this and so was prevented from regenerating.

Case V

The tail of this tadpole had suffered the same kind of injury as in the previous three cases, but the incision had been made from the ventral edge of the tail upward. The resulting accessory tail (Fig. 5) was similar to those described above. The incision had been deep enough to sever the nerve cord, as well as the notochord, for it had regenerated and appeared in the new tail

and also in the old tail end. Again, the notochord was present in the accessory tail together with some muscle fibers. The caudal vessels were present and more nearly normal than in any of the other cases.

Discussion and Conclusions

In a subsequent paper the role of the notochord and of the nerve cord in the regeneration of the tail of the frog tadpole will be discussed, particularly with respect to experimentally produced accessory tails. It has been shown that incomplete amputation of the tails of lizards and urodeles permits the production of accessory tails by regeneration. Similarly, the occurrence of accessory tails described above demonstrates that, if the tail of the *Rana clamitans* tadpole is incompletely amputated, or injured, in such a manner as to leave the old tail end attached to the stump and also to provide a surface for regeneration of the notochord, an accessory tail results. Since the structure of the tail is practically identical in the various species of frog larvae it may be assumed that this phenomenon could occur in other species, especially in those that have relatively long aquatic lives. It is significant that, in each of these five cases, the notochord had been severed, and that in two cases the nerve cord had not been injured. The nerve cord appeared in the accessory tail only when it had been severed by the injury that made the accessory tail possible.

Acknowledgment

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